THE ROLE OF LATENT MEMBRANE PROTEIN 2 IN THE PATHOGENESIS OF EPSTEIN-BARR VIRUS ASSOCIATED MALIGNANCY

By

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Date 08/01/2007

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ABSTRACT

Epstein-Barr virus (EBV) establishes a lifelong latent infection in host B cells and is associated with the development of various lymphoid and epithelial malignancies. Approximately half of Hodgkin's lymphomas (HL) are EBV positive where LMP2 is highly expressed. However, the contribution of LMP2 to the pathogenesis of HL remains largely unknown. In this study, HL cell lines stably expressing LMP2A and LMP2B HL were generated. The impact of global gene expression in KMH2 cells expressing LMP2A and LMP2B was performed using Gene expression microarrays; this identified a number of potentially important transcriptional targets of LMP2A and LMP2B which may be of relevance to the development of HL, but also highlighted important difference in the effect of these proteins on cellular gene expression that were dependant on their expression levels.

In addition, to identify cellular proteins that interact with LMP2, His-tagged versions of LMP2A and LMP2B were stably expressed in HEK 293 cells. The expression of LMP2 in these cells was confirmed. A new approach stable isotope labelling amino acid in cell culture (SILAC) to identify interacting proteins which combined with LC-MS/MS analysis was performed and 10 novel LMP2A interactors and 20 novel LMP2B interactors were identified. All these potential interactions require validation but of particular interest is the possible interaction of DNA methyl transferase 1 (DNMT1) with LMP2A and OB-cadherin with LMP2B.

Overall, the studies described in this thesis demonstrate that LMP2A and LMP2B have profound effects on the behaviour of HL cells and SILAC combined with LC-MS/MS is a useful and reliable approach to identify interacting proteins.

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Abbreviation

AICDA or AID	Activation-induced cytidine deaminase
AIDS	acquired immunodeficiency syndrome
AMV	avian myeloblastosis
Bala	NPC patient reference serum
BARF0	BamHI A rightward frame 0
BARTs	BamHI A rightward transcripts
BCR	B cell receptor
BL	Burkitt's Lymphoma
BLNK	B-cell linker
bp	base pair
Btk	Bruton's tyrosine kinase
cDNA	complementary deoxyribonucleic acid
cHL	classical Hodgkin's lymphoma
Csk	c-Src kinase
CTAR	C-terminal activating region
CTL	cytotoxic T lymphocyte
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EA	early antigen
EAP	EBER associated protein
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	diaminoethanetetra-acetic acid
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FCS	foetal calf serum
FITC	fluorescence isothiocyanate
FL	follicular lymphoma
FT	Flow-through fractions
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	germinal centre
GCOS	GeneChip Operating Software
Gp	glycoprotein
HHV	human herpesvirus
His	histidine
HIV	human immunodeficiency virus
HL	Hodgkin's lymphoma
HLnlp	nodular lymphocyte-predominant Hodgkin lymphoma

HPV	Human papillomavirus
HRP	Horseradish Peroxidase
HRS	Hodgkin's Reed Sternberg cells
IF	Immunofluorescence staining
IFI27	Interferon, alpha-inducible protein 27
IFN	Interferon
IM	infectious mononucleosis
IR	internal repeat
ISGs	interferon-stimulated genes
ITAM	Immunoreceptor tyrosine-based activation motif
IVT	in vitro transcription
JAK	janus kinase
JNK	c-jun N-terminal kinase
Kbp	kilobase pair
Kda	kilodalton
LCL	lymphoblastoid cell line
LC-MS	liquid chromatography mass spectrometry
LMP	latent membrane protein
m/z	mass to charge ratio
MAPK	mitogen activated protein kinase
MC	mixed cellularity
MH+	mass of ion (M: molecule; H+, proton)
MHC	major histocompatibility complex
MS	Mass Spectrometry
MS/MS	tandem mass spectrometry
NF-κB	nulear factor kappa B
Ni-NTA	nickel nitrilotriacetic acid
NK	natural killer
NPC	nasopharyngeal carcinoma
NS	nodular sclerosis
ORF	open reading frame
PAL	pyothorax-associated lymphoma
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBST	PBS-tween buffer
PCR	polymerase chain reaction
pfp	percentage of false-positives
PFA	Paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PLC-γ2	phospholipase C-γ2
PTK	protein tyrosine kinase
PTLD	post transplant lymphoproliferative disease

PY	phosphotyrosine
R	[12C6]-Arginine
R#	[13C6]-Arginine
RAG	recombinase activating genes
RBP-Jk	recombination binding protein J kappa
RMA	robust multi-array analysis
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT	reverse transcription
SAP	Shrimp Alkaline Phosphatase
SDS	sodium dodecyl sulphate
Ser	serine
SH2	Src homology 2
SHM	somatic hypermutation
SILAC	Stable isotope labelling with amino acids in cell culture
STAT	signalling transducer and activator of transcription
TCR	T cell receptor
Thr	threonine
TR	terminal repeat
TRADD	TNF receptor associated death domain protein
TSG	tumour suppressor gene
UL	unique long
US	unique short
VCA	viral capsid antigen
XC	XCorrection score
Y	tyrosine

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CHAPTER ONE: INTRODUCTION

1 Introduction

1.1 Epstein-Barr virus (EBV)

The Epstein-Barr virus (EBV), also called *Human herpesvirus 4* (HHV-4), is a virus of the herpes family and was identified as the first candidate human tumour virus due to its consistent association with African Burkitt's lymphoma (Epstein *et al.*, 1964, Epstein *et al.*, 1965; Henle and Henle, 1966). It is named after Epstein and Barr, who, along with B.G. Achong, discovered the virus in 1964 in a cultured Burkitt's lymphoma cell line (Epstein *et al.*, 1964). EBV is widespread in more than 90% of all human populations (Henle *et al.*, 1969; Henle *et al.*, 1976; Henle and Henle, 1979); most people carry EBV asymptomatically. EBV persists in the infected host as a latent infection in B lymphocytes and as probably a lytic infection in the oropharynx (Nilsson *et al.*, 1971; Gerber *et al.*, 1982). EBV was shown to transform resting B lymphocytes in vitro leading to the outgrowth of B lymphoblastoid cell lines (LCLs), thus establishing EBV as B lymphotropic transforming virus (Henle *et al.*, 1967). EBV is associated with certain haematopoietic cancers, such as Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL) and T-cell and NK-cell lymphomas (Rickinson and Kieff, 2001). It is also associated with two epithelial origin diseases, nasopharyngeal carcinoma (NPC), oral hairy leukoplakia and post-transplant lymphoproliferative disease (PTLD) (Rickinson and Kieff, 2001). All these EBV related diseases will be detailed later.

1.2 EBV classification

EBV is a member of the *Herpesviridae*. This family of viruses can be further divided into three subfamilies: alpha, beta and gamma. The subdivision was originally made according to biological properties such as host range, duration of

reproductive cycle, cytopathology and characteristics of latent infection but is now at the level of genome organisation and homology. The gamma herpesvirus subfamily is subdivided into two genera, gamma 1 or lymphocryptovirus (LCV), and gamma 2 or rhadinovirus (RDV). EBV is the prototype LCV and the only human virus in the genus. Many Old World primate species and some New World primate species have their own LCVs but such viruses are not found in non-primate hosts. LCV are predominantly B lymphotropic and although latent in B cells in vivo they can also reactivate to produce lytic infection. Whether there is a non-B cell type, which is naturally fully permissive for LCVs in vivo remains in doubt, but it is possible that an epithelial cell of this kind exists in the oropharynx and is the source of chronic viral shedding.

1.3 EBV structure

EBV has a 172 kilobase pair (kb) linear, double-stranded DNA genome wrapped around a toroid-shaped protein core, encased by a nucleocapsid and surrounded by a glycoprotein (gp)-rich envelope (Epstein *et al.*, 1965). Initially the genome was divided into short and long unique domains, (US and UL), either side of the reiterated 3kbp internal repeats, IR1 (Hayward *et al.*, 1982). UL is further subdivided by repeats IR2-4 into US2-5. The ends of the linear genome are flanked by 4-12 copies of tandem, reiterated, 500bp direct repeats, termed terminal repeats (TRs), (Hayward and Kieff, 1976; Kintner and Sugden, 1979), which mediate circularisation of the viral DNA into episomes. This feature enables the cell clonality of viral episomes to be establised, as latently infected progeny cells will retain the same number of TRs as the parental cell (Raab-Trauband and Flynn, 1986), although a recent study reported that TR number

varied inversely to the quantity of LMP2A transcripts for both lymphoid and epithelial cell types *in vitro* (Moody *et al.*, 2003).

EBV was the first herpesvirus to have its complete genome cloned and sequenced (Dambaugh *et al.*, 1980; Arrand *et al.*, 1981; Biggin *et al.*, 1984). This was done using *Bam*HI restriction fragments from the B95.8 EBV strain and consequently the nomenclature was changed to reflect this cloning strategy (Biggin *et al.*, 1984). Every cell in an LCL carries multiple copies of the viral episome and constitutively expresses a limited set of viral gene products, the so-called latent proteins, consisting of six nuclear antigens (EBNAs1, 2, 3A, 3B, 3C and -LP) and three latent membrane proteins (LMPs1, 2A and 2B) (Kieff and Rickinson, 2001). EBNA-LP is transcribed from a variable number of repetitive exons. LMP2A and LMP2B are composed of multiple exons, which are located on either side of the terminal repeat (TR) region, which is formed during the circularization of the linear DNA to produce the viral episome. EBER1 and EBER2 are highly transcribed non-polyadenylated RNAs and their transcription is a consistent feature of latent EBV infection. All the EBNAs are transcribed from either the Cp or Wp promoter; the different EBNAs are encoded by individual mRNAs that are generated by differential splicing of the same long primary transcript. The *Bam*HI restriction-endonuclease map of the prototype B95.8 genome shows that the *Bam*HI fragments are named according to size, with A being the largest and lowercase letters being the smallest fragments. Figure 1.1 shows a schematic of the EBV genome.

Open reading frames (ORFs) are stated relative to their *Bam*HI fragment, direction of transcription and position relative to other ORFs located on the same fragment. For example, BHRF1 is the first rightward ORF located in the *Bam*HI

H fragment. All EBV strains have essentially the same genome organisation and the sequences are highly conserved overall. However, sequence analysis of different geographic EBV isolates had revealed two types, type 1 and 2, which differ primarily in the latent genes encoding EBV nuclear antigens (EBNAs)-LP, 2, 3A, 3B and 3C (Sixbey *et al.*, 1989; Sample *et al.,* 1990). EBV strains of the same type can be further classified according to local changes at polymorphic sites, such as the number of repeat sequences in several of the latent genes, the presence or absence of a 30bp region within the LMP1 gene, as well as single nucleotide changes particularly in the genes encoding EBNA1, EBNA2 and LMP1 (Lung *et al.*, 1994; Miller *et al.*, 1994; Falk *et al.*, 1995; Khanim *et al.*, 1996; Sandvej *et al.*, 1997). Many of these local polymorphisms distinguish EBV strains of different geographic origins, consistent with a slow evolutionary drift of the virus over thousands of years in physically separate human populations. However, the origins of the two different EBV types, where EBNA2, 3A, 3B and 3C alleles with 47%, 16%, 20% and 28% nucleotide homology at the respective loci are found within genomes that are otherwise highly homologous, remains a mystery. Epidemiologically, type 1 virus strains are dominant in most populations but in equatorial Africa and New Guinea, areas where BL is endemic, type 2 strain are nearly as prevalent as type 1 viruses (Young *et al.*, 1987; khanim *et al.*, 1996; Yao *et al.*, 1998; Habeshaw *et al.*, 1999).

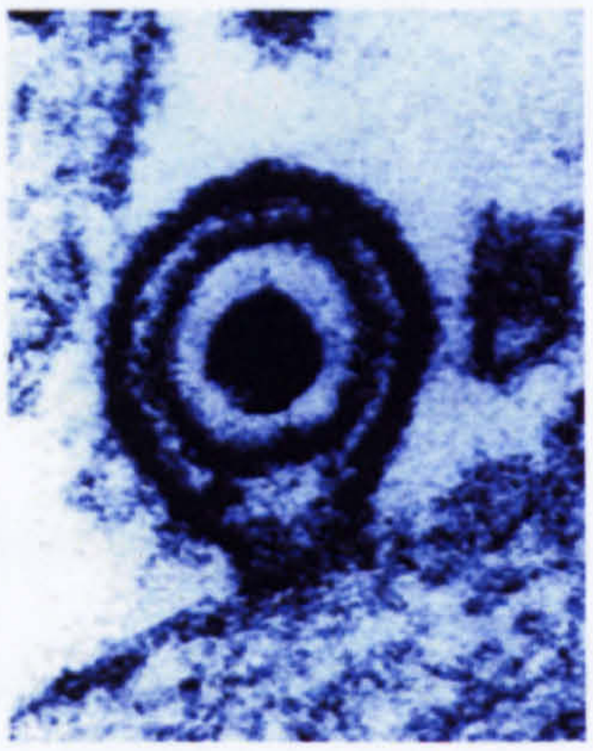
Figure 1-1 Schematic of the Epstein-Barr virus

A: Electron micrograph of the Epstein–Barr virus (EBV) virion.

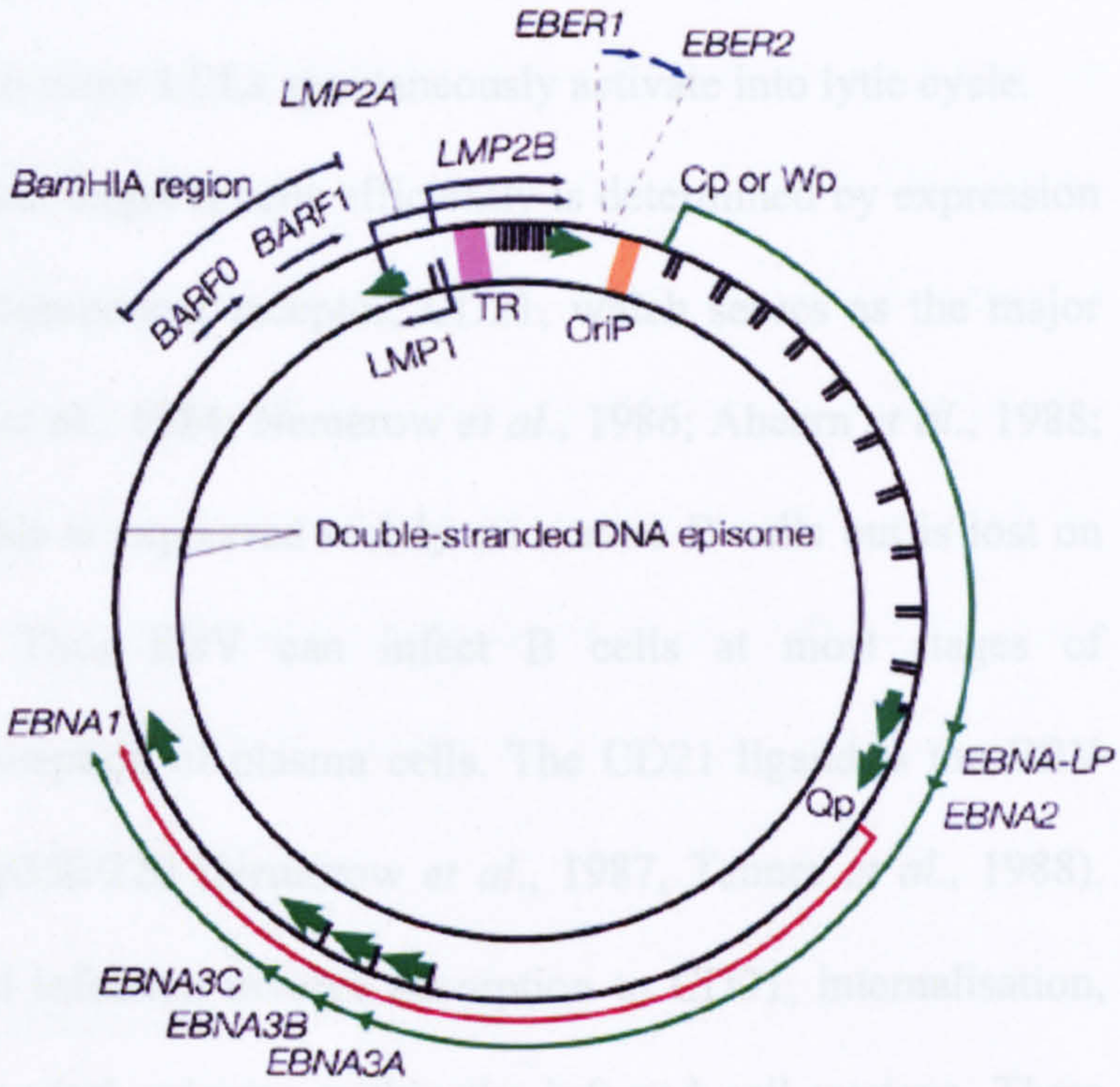
B: Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The origin of plasmid replication (OriP) is shown in orange. The large green solid arrows represent exons encoding each of the latent proteins, and the arrows indicate the direction in which the genes encoding these proteins are transcribed. *LMP2A* and *LMP2B* are composed of multiple exons, which are located on either side of the terminal repeat (TR) region. The blue arrows at the top represent the highly transcribed non-polyadenylated RNAs *EBER1* and *EBER*. The long outer green arrow represents EBV transcription during a form of latency known as latency III, in which all the EBNAs are transcribed from either the Cp or Wp promote. The inner, shorter red arrow represents the *EBNA1* transcript, which originates from the Qp promoter during Lat I and Lat II. The locations of the BARF0 and BARF1 coding regions are shown here.

C: Location of open reading frames for the EBV latent proteins on the *Bam*HI restriction-endonuclease map of the prototype B95.8 genome.

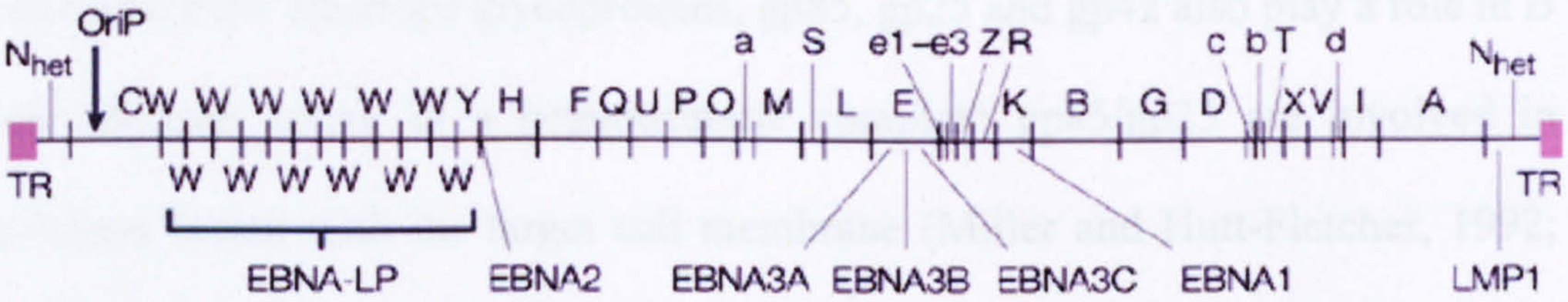
a EBV electron micrograph



b EBV genome: latent genes



c Open reading frames for the EBV latent proteins



Taken from Young and Rickinson (2004). Nature Reviews

1.4 EBV tissue tropism

When tested on human target cells in vitro, efficient EBV infection is restricted to B lymphocytes and results in their transformation to continuously proliferating B LCLs (Henle *et al.*, 1967). Such LCLs express a small subset of viral genes, now called the latent genes, a pattern of expression termed Latency III. In addition a small percentage of cells in many LCLs spontaneously activate into lytic cycle.

The ability of EBV to infect target B cells efficiently is determined by expression of the C3d complement component receptor, CD21, which serves as the major virus receptor (Fingeroth *et al.*, 1984; Nemerow *et al.*, 1986; Ahearn *et al.*, 1988; Nemerow *et al.*, 1990); this is expressed widely on mature B cells but is lost on terminal differentiation. Thus EBV can infect B cells at most stages of development, with the exception of plasma cells. The CD21 ligand is the EBV outer envelope protein gp350/220 (Nemerow *et al.*, 1987, Tanner *et al.*, 1988). The initial events of viral infection involve adsorption to CD21, internalisation, and circularisation of the viral episome within the infected cell nucleus. Three additional EBV envelope glycoproteins, gp85, gp25 and gp42 also play a role in B cell infection acting as a heterotrimeric complex; gp85/gp25 are involved in envelope fusion with the target cell membrane (Miller and Hutt-Fletcher, 1992; Haddad and Hutt-Fletcher, 1989) and gp42 in co-receptor interactions by engaging human leukocyte antigen (HLA) class II molecules that are naturally expressed on the B cell surface (Wang and Hutte-Fletcher, 1998; Molesworth *et al.*,2000; Ressing *et al.*, 2005).

EBV can also establish infections in other cell types, as shown by the association between EBV and epithelial or T cell malignancies. However, it is not known what mode of entry EBV utilises to infect these cell types. Certainly in vitro

infection of primary epithelial and T cells, where possible, is very inefficient (Groux *et al.*, 1997) and does not result in transformation (Yoshiyama *et al.*, 1997; Imai *et al.*, 1998; Fingeroth *et al.*, 1999). Immature T cell populations transiently express very low levels of CD21 during thymic development and in vitro infection of these cells has been claimed (Tsoukas and Lambris, 1993). However, reports of in vitro infection of CD21-negative T cells first suggested the possibility of a gp350/CD21-independent route (Hedrick *et al.*, 1992). This is now confirmed by the fact that recombinant gp350-knockout viruses can infect a variety of cell types; infection is nevertheless much less efficient than that seen by wild-type virus in B cells. It is thought that gp85/gp25 complexes may be important in CD21-independent infection (Molesworth *et al.*, 2000; Oda *et al.*, 2000) as well as gp110 (Neuhierl *et al.*, 2002); the ligand for gp110 is as yet unknown.

1.5 Patterns of latent infection

1.5.1 Latency I

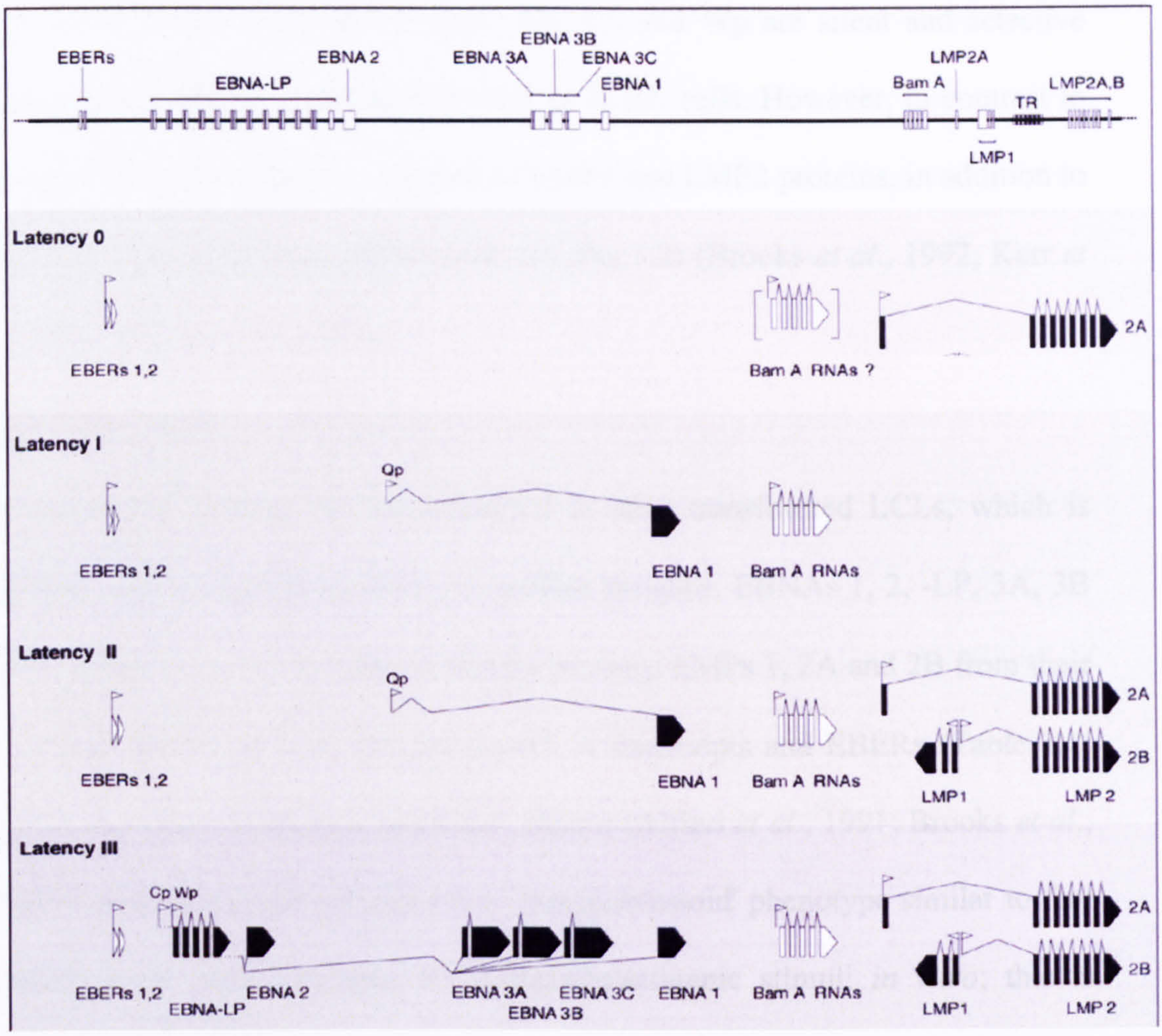
Burkitt's lymphoma (BL) is characterised by a Latency I pattern (Gregory *et al*, 1990). In latency I infected cells the EBNA promoters Cp and Wp, and the LMP promoters are silent, but there is activation of an alternative EBNA promoter, termed Qp, located downstream in the *Bam*HI Q fragment (Schaefer *et al.*, 1995; Nonkwelo *et al.*, 1996). EBNA1 is the only latent protein to be expressed from Qp and is encoded by a novel Q-U-K spliced transcript. The non-coding EBER RNAs and *Bam*HI A transcripts are also detectable in Latency I cells (Table 1.1, Figure. 1.2) (Rowe *et al.*, 1987; Brooks *et al.*, 1993).

Table 1-1 Pattern of EBV-latent gene expression and the cell types in which these patterns occur

Type of Latency	Latent genes expressed	Cell types
Latency 0	EBERs, LMP2A *Bam*HI A, EBNA1?	Resting B cells *in vivo*
Latency I	EBNA1, EBERs, *Bam*HI A	BL tumour cells, most early passage BL cell lines.
Latency II	EBNA1, LMP1, LMP2, EBERs, *Bam*HI A	Epithelial cells from NPC and Reed-Sternberg cells in HL
Latency III	EBNAs 1, 2, 3a, 3b, 3c,-LP, LMP1,LMP2, EBERs, *Bam*HI A	LCLs in vitro, IM and PTLD tumour cells *in vivo*. Some unstable BL cell lines may also drift towards this phenotype.

Figure 1-2 Pattern of EBV-latent gene transcription in three different forms of latency

Transcripts are drawn in correct orientation relative to one another, with promoter (pennants) and splicing patterns as shown; exons are illustrated for non-coding RNAs (white) and for mRNA (black). The actual genomic positions of those exons are shown (top) on a linear map of the viral genome. Burkitt's lymphoma (BL) is characterised as Latency I (Gregory *et al*, 1990). Hodgkin's lymphoma (HL) and Nasopharyngeal Carcinoma (NPC) both exhibit the Latency II pattern of gene expression (Young and Murray, 2003) while Post-Transplant Lymphoproliferative Disease (PTLD) shows Latency III (Young and Murray, 2003).



Taken from Prof. Kieff and Rickinson (2001). Virology, 4th Edition

1.5.1 Latency II

Another restricted form of latency, termed latency II, is observed in the Reed-Sternberg cells of EBV-positive Hodgkin's lymphoma (HL), and in the epithelial cell tumour nasopharyngeal carcinoma (NPC) (Young and Murray, 2003; Pallesen *et al.*, 1991). In Latency II infected cells, Cp and Wp are silent and selective expression of EBNA 1 initiates from Qp, as in BL cells. However, in contrast to Latency I, there is variable expression of LMP1 and LMP2 proteins, in addition to *Bam*HI A and EBER transcripts (Table 1.1; Fig.1.2) (Brooks *et al.*, 1992; Kerr *et al.*, 1992; Deacon *et al.*, 1993).

1.5.2 Latency III

The pattern of Latency III was observed *in vitro* transformed LCLs, which is characterised by expression of all six nuclear antigens, EBNAs 1, 2, -LP, 3A, 3B and 3C from Cp or Wp and the membrane proteins LMPs 1, 2A and 2B from their own promoters along with spliced *Bam*HI A transcripts and EBERs (Table 1.1; Fig.1.2 taken from (Rickison and Kieff, 2001)) (Alfieri *et al.*, 1991; Brooks *et al.*, 1993). Latency III infection induces a ' lymphoblastoid' phenotype similar to that observed in B cells following by mitogenic/antigenic stimuli *in vitro*; this is associated with high level expression of activation and adhesion markers, HLA molecules and the acquisition of efficient antigen-processing function (Peng and Lundgten, 1993; Rowe *et al.*, 1995).

Latency III transcripts are also detectable in peripheral blood mononuclear cells (PBMCs) and tonsils of infectious mononucleosis (IM) patients, indicating a similar growth transforming program is induced during primary infection in vivo (Tierney *et al.*, 1994; Niedobitek *et al.*, 1997). However, in immunocompetent individuals this form of latency elicits a strong $CD8^+$ cytotoxic T cell (CTL)

immune response such that Latency III transcripts are no longer detectable in the blood of healthy virus carriers, although they may be detected in tonsillar B cell preparations as a result of new EBV infections initiated from lytically-infected cells releasing progeny virus in the oropharynx (Babcock *et al.*, 2000). The importance of the immune system in controlling EBV infections is underlined in the case of immunocompromised transplant patients where Latency III infected cells are often detected in the proliferating tumour cells of EBV-positive post-transplant lymphoproliferative disease (PTLD) (Young and Murray, 2003; Young *et al.*, 1989).

1.5.3 Latency 0

A fourth form of latency, termed Latency 0 has been proposed to account for EBV persistence in resting memory B cells *in vivo* (Babcock *et al.*, 1999); this would be analogous to that seen in latent alpha herpesvirus infections of neuronal cells *in vivo*. In Latency 0, viral transcription is limited to EBER, LMP2A and possibly the EBNA1 and BamHI A transcripts (Miyashita *et al.* 1995; Miyashita *et al.* 1997) (Table1.1; Fig.1.2).

1.6 EBV latent antigens

1.6.1 EBNA1

EBV-infected cells express a group of nuclear proteins that influence both viral and cellular transcription. EBNA1 is expressed in all virus-infected cells, in which its role is the maintenance and replication of the episomal EBV genome, achieved through sequence-specific binding to the plasmid origin of viral replication, OriP (Kieff and Rickinson, 2001) (Figure 1.1 b,c). EBNA1 can also interact with certain viral promoters, thereby contributing to the transcriptional regulation of the EBNAs (including EBNA1 itself) and of LMP1. EBNA1 is separated into

amino- and carboxy-terminal domains by a Gly-Ala repeat sequence, the main function of which seems to be to stabilize the mature protein — preventing its proteasomal breakdown (Levitskaya *et al.*, 1995) — rather than functioning in its originally suggested role as an immune-evasion domain (Voo *et al.*, 2004; Lee *et al.*, 2004; Tellam *et al.*, 2004). Gene-knockout studies indicate that EBNA1 does not have a crucial function in *in vitro* B-cell transformation beyond the maintenance of the viral genome (Humme *et al.*, 2003); on the other hand, a more direct involvement in oncogenesis is indicated by the ability of B-cell-directed EBNA1 expression to produce B-cell lymphomas in transgenic mice (Wilson *et al.*, 1996), and by its possible contribution to the survival of Hodgkin's lymphoma cells (Hammerschmidt and Sugden, 2004).

EBNA1 is the only viral protein that is consistently expressed in all EBV-associated malignancies, as well as in chronic active EBV infection (Yoshioka *et al.*, 2003), and is essential for viral DNA replication and maintenance of the viral episomes in infected cells. EBNA1 binds the symmetrical sequences of the origin of replication of the viral DNA and together with cellular proteins co-ordinates replication of viral episomes with cellular DNA (Yates *et al.*, 1985). Both LMP1 and EBNA1 are regulated by the JAK-STAT (Janus kinase-signal transducers and activators of transcription) signalling transduction pathways, suggesting that dysregulation of the JAK-STAT pathway may precede the development of EBV-associated tumorigenesis (Chen *et al.*, 2001).

1.6.2 EBNA2

EBNA2 is a transcriptional transactivator that is one of the first viral genes expressed after EBV infection of B cells and is essential for EBV immortalization of B cells in vitro (Cohen *et al.*, 1989; Hammerschmidt *et al.*, 1989). The inability

of the P3HR-1 EBV, which has deletion encompassing EBNA2 and the last two exons of EBNA-LP, to transform B cells *in vitro* was the first indication of the crucial role of EBNA2 in the transformation process (Kieff and Rickinson, 2001). Restoration of the EBNA2 gene in P3HR-1 cells has unequivocally confirmed the importance of EBNA2 in B-cell transformation and has allowed the functionally relevant domains of EBNA2 to be identified (Hammerschmidt and Sugden, 1989; Cohen *et al.*, 1989). EBNA2 interacts with a sequence-specific DNA-binding protein, Jκ-recombination-binding protein (RBP-Jκ), to transcriptionally activate cellular genes such as *CD23* and the key viral genes *LMP1* and *LMP2A* (Kieff and Rickinson, 2001; Grossman *et al.*, 1997; Hsieh *et al.*, 1995). EBNA2 is a mimic of NotchIC (Zhang *et al.*, 2001; Zimber-Strobl *et al.*, 2001). Although the role of Notch in the B cell compartment remains incompletely understood, recent observations allow speculation on the ways in which the EBNA2 usurping of Notch function may sustain a life-long EBV infection in the host (Zimber-Strobl *et al.*, 2001; Izon *et al.*, 2002; Radtke *et al.*, 2002). The c-*MYC* oncogene also appears to be an important target of EBNA2 and this effect deems to be important for EBV-induced B cell proliferation (Kaiser *et al.* 1999).

1.6.3 The EBNA3 Family

An essential role for EBNA3A and EBNA3C in B-cell transformation *in vitro* has been shown using EBV recombinants, whereas EBNA3B is dispensable (Tomkinson *et al.*, 1993). EBNA3C can cooperate with RAS in rodent-fibroblast transformation assays and disrupt cell-cycle checkpoints (Parker *et al.*, 1996; Parker *et al.*, 2000). These effects are partly explained by the interaction of EBNA3C with factors that modulate transcription (for example, histone deacetylase 1, nonmetastatic protein 23-homologue 1 and C-terminal binding

protein) or influence cell-cycle progression (for example, cyclin A) (Radkov *et al.*, 1999). EBNA3C can induce the up-regulation of both cellular (CD21) and viral (LMP1) gene expression can repress the Cp promoter (Allday and Farrell, 1997; Radkov *et al.*, 1997) and might interact with the retinoblastoma protein pRb to promote transformation (Parker et al., 1996). EBNA3B has been shown to induce expression of vimentin and CD40 (Silins and Sculley, 1994)

1.6.4 EBNA-LP

EBNA-LP interacts with EBNA2 and is required for the efficient outgrowth of virus-transformed B cells *in vitro* (Mannick *et al.*, 1991; Sinclair *et al.*, 1994). The transcriptional activation, which is mediated by EBNA2 in conjunction with EBNA-LP is modulated by the EBNA3 family of proteins, which repress transactivation (Robertson *et al.*, 1996; Zhao *et al.*, 1996). EBNA-LP and EBNA2 were transfected transiently into primary B cells and induced transition from G_0 to G_1 as measured by the up-regulation of cyclin D_2 expression (Yokoyama *et al.*, 2001). EBNA-LP can also cooperate with EBNA2 in upregulating transcriptional targets of EBNA2, including LMP1 (Nitsche *et al.*, 1997, MacCann *et al.*, 2001).

1.6.5 LMP1

LMP1 is a 66kDa integral membrane protein encoded by the BNLF1 ORF, and consists of a short N-terminal cytoplasmic tail of 17 amino acid residues, six hydrophobic transmembrane segments and a long C- terminal cytoplasmic region of 200 amino acid residues (Fennewald *et al.*, 1984). After translation, LMP1 inserts into the plasma membrane, where it aggregates in tight plasma membrane patches (Liebowitz *et al.*, 1986). Aggregation is essential for LMP1 signalling and is dependent upon the presence of the transmembrane domains (Baichwal and Sugden, 1989; Liebowitz *et al.*, 1992). Vimentin co-localises with LMP1 and is

drawn into the patch by LMP1 but once associated can redirect LMP1 to other sites (Liebowitz *et al.*, 1987). However, the functional significance of this association is unclear as LMP1 can still transduce signals in B cell lines that lack vimentin (Liebowitz and Kieff, 1989). Approximately 30% of LMP1 assembles in glycosphingolipid (GSL) rafts and this localisation may be important for its function (Clausse et al., 1997; Ardila-Osorio *et al.*, 1999). LMP1 has a relatively short half-life of 30 minutes, extending to 2-3 hours when phosphorylated on serine and threonine residues (Mann and Thorley-Lawson, 1987; Moorthy and Thorley-Lawson, 1990).

LMP1 is essential for primary B cell transformation (Baichwal and Sugden, 1988; Kaye *et al.*, 1993) and the sustained growth of LCLs (Kaye *et al.*, 1993; Kilger *et al.*, 1998). Indeed, it is the best example of an EBV protein that is active on its own in conventional assays of transforming function. The expression of LMP1 in the B lymphocytes of transgenic mice predisposes them to develop lymphoma late in life (Kulwichit *et al.*, 1998). LMP1 is also capable of transforming Rat-1 fibroblasts *in vitro*, inducing morphology changes, anchorage independent growth, loss of contact inhibition and tumourigenicity in nude mice (Baichwal and Sugden, 1989). For these reasons, LMP1 is generally considered to be an oncogenic protein.

In addition to the transmembrane domains, two regions spanning amino acid residues 185-231 and amino acid residues 351-386, within the C-terminal cytoplasmic domain, termed (carboxy terminus activating regions) CTAR1 and CTAR2 respectively, play critical roles in the transforming phenotype of LMP1 (Izumi and Kieff, 1997; Kaye *et al.*, 1999). Deletion of CTAR1 shows it is essential for primary B cell transformation (Izumi *et al.*, 1997; Kaye *et al.*, 1999);

deletion of CTAR2 did not fully abrogate transformation but severely impaired growth of the resulting LCLs (Izumi and Kieff, 1997).

LMP1 mediates cellular phenotypic changes by stimulating signal transduction pathways that lead ultimately to the activation of transcription factors, such as nuclear factor kappaB (NF-kB) and activating protein-1 (AP-1) (Eliopoulos and Young, 1998; Hatzivassiliou *et al.*, 1998). LMP1 expression in EBV-negative B cells results in the upregulation of activation markers CD21, CD23, CD30, CD39, CD40 and CD44 and of cell adhesion molecules ICAM1, LFA1 and LFA3 and can even restore antigen processing function to BL cells (Peng and Lundgren, 1993; Huen *et al.*, 1995; Rowe *et al.*, 1995). LMP1 expression in epithelial cells induces epidermal growth factor receptor (EGFR) expression (Miller *et al.*, 1995b). In B cells and epithelial cells LMP1 can induce anti-apoptotic factors, A20 and Bcl2 (Laherty *et al.*, 1992; Rowe *et al.*, 1994).

As the cytoplasmic tail of LMP1 does not possess intrinsic kinase activity istself, it is thought to exert its effects through association with tumour necrosis factor (TNF) receptor associated factors (TRAFs). LMP1 resembles members of the TNF receptor family and can in part functionally substitute for CD40 in *in vitro* assays of CD40 signalling (Eliopoulos *et al.*, 1997; Floettmann *et al.*, 1998; Hatzivassiliou *et al.*, 1998; Kilger *et al.*, 1998) and can even mimic some (but not all) of CD40 functions *in vivo* in a transgenic mouse model (Uchida *et al.*, 1999).

There are two recognized NF-kB pathways: the canonical pathway which mainly utilizes IKK-β to phosphorylate IKB-α resulting in the generation of p50/p65 dimer; and the non-canonical pathway, which involves IKK-α leading to the processing of p100 (NF-kB-2) and generation of p52/ReIB dimers. Recently, it has been shown that the CTAR1 domain of LMP1 activates NF-kB mainly though

the non-canonical pathway (TRAF3/NIK/IKK-α) (Brodeur *et al.*, 1997; Sandberg *et al.*, 1997; Eliopoulos *et al.*, 1999; Lufitig et al., 2003). In contrast, CTAR2 appears to activate the canonical pathway by utilizing TRAF6 and TAK1 to activate IKK-β (Wu *et al.*, 2006). It also appears that much of LMP1-induced NF-kB activation is TRADD-independent (Wu *et al.*, 2006). CTAR2 is also important in LMP1 activation of the c-Jun N-terminal kinase (JNK) pathway leading to activation of AP-1 (Eliopoulos and Young, 1998; Hatzivassiliou *et al.*, 1998; Eliopoulos *et al.*, 1999a). CTAR1 and CTAR2 can both induce p38 MAPK activation in HEK 293 and Rat-cells leading to activation of the transcription factor ATF2 (Eliopoulos *et al.*, 1999b).

The region of LMP1 lying between CTAR1 and CTAR2, sometimes referred to as CTAR3, is not required for EBV immortalisation of B lymphocytes (Izumi *et al.*, 1999a) but has been implicated in activating the janus kinase/signalling transducer and activator of transcription (JAK/STAT) pathway via JAK3 binding (Gires *et al.*, 1999). However, mutation of this site does not affect JAK signalling implying some redundancy of function. LMP1 has also been shown to signal through GTPases such as Cdc42, inducing reorganisation of the cytoskeleton in serum-starved mouse fibroblasts (Puls *et al.*, 1999). The expression of LMP1 in NPC is associated with increased metastatic spread, an effect that is also reflected in the ability of LMP1 to induce increased cell motility and invasive growth when expressed in epithelial cells *in vitro* (Kim *et al.*, 2000; Horikawa *et al.*, 2000; Horikawa *et al.*, 2001). LMP1 can activate the PI3K/Akt pathway and this effect is responsible for LMP1-induced actin polymerization, morphological transformation, and cell survival (Dawson *et al*, 2003).

1.6.6 LMP2

1.6.6.1 The structure of LMP2A and LMP2B

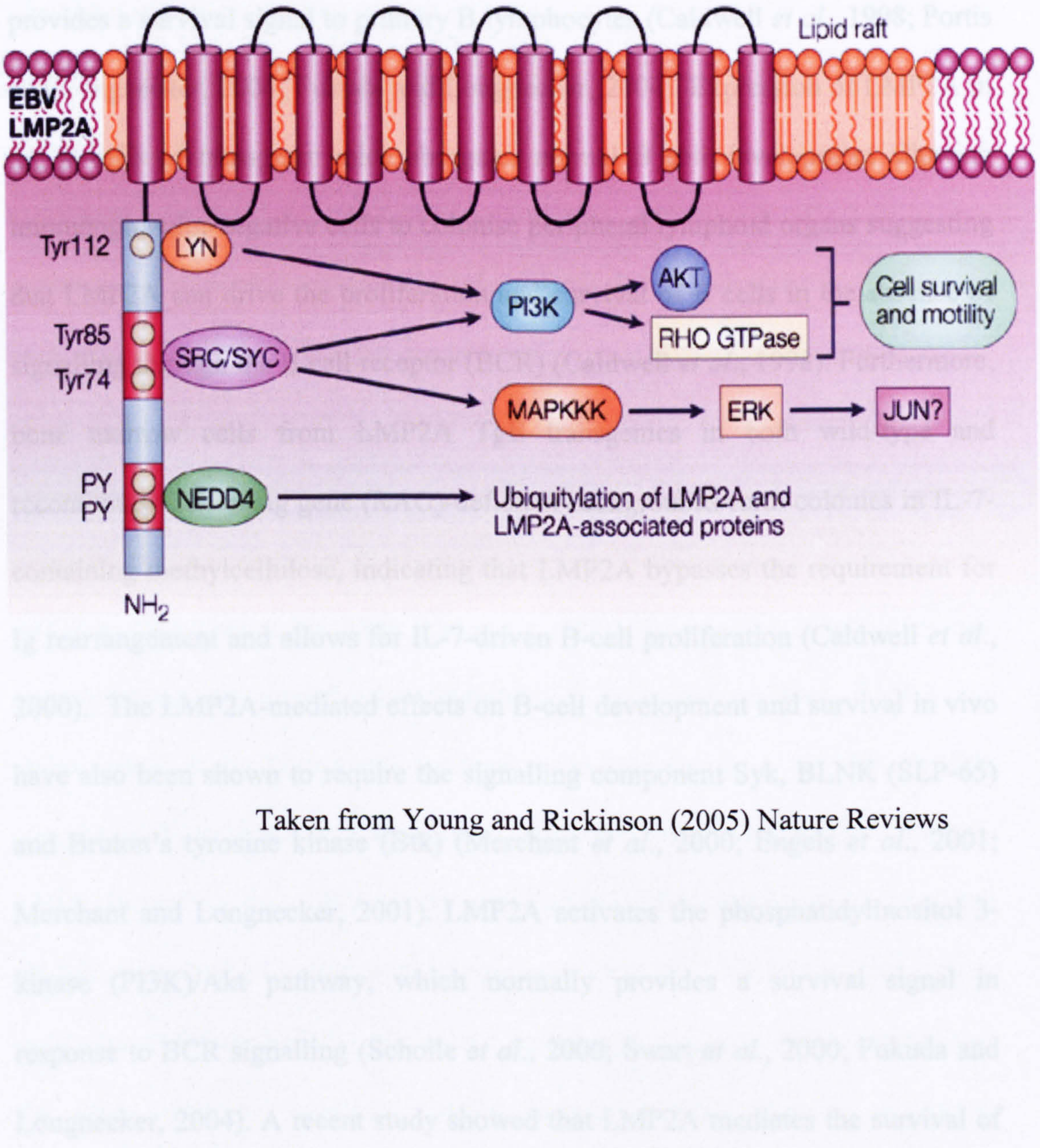
There are two LMP2 proteins, LMP2A and LMP2B which are encoded by 2.0kb and 1.7kb mRNAs messages respectively, which initiate from promoters 3kb apart in the EBV genome and splice across the TRs (Hudson *et al.*, 1985; Laux *et al.*, 1988; Laux *et al.*, 1989; Sample *et al.*, 1989). Each of the transcripts has a unique first exon but 8 shared common exons. The first exon of LMP2A encodes a 119-amino-acid N-teminal cytoplasmic domain unique to this protein. The first exon of LMP2B is non-coding and translation of LMP2B initiates at a methionine codon at the beginning of the common second exon (Sample *et al.*, 1989). The remaining LMP2A and LMP2B exons encode 12 hydrophobic integral membrane-spanning domains and a 27-amino-acid hydrophilic cytoplasmic C terminal tail (Figure1.3) (Longnecker, 2000). Thus, the predicted molecular weights of LMP2A and LMP2B are 54kDa and 40kDa, respectively. The hydrophobic domains led to speculation that the LMP2 is a membrane associated protein, and this has been confirmed. However the exact subcellular localisation remains controversial. It was reported that LMP2A localised to the same patch of the plasma membrane as LMP1 in latently infected B cells (Longnecker and Kieff, 1990). LMP2A localized to the same site in the plasma membrane of B-lymphoma cells whether or not LMP1 was expressed in the same cell (Longnecker and Kieff, 1990). However, other studies reported a distinct intracytosolic pattern of LMP2 distribution (Lynch *et al.*, 2002). In non-B-cells, LMP2A and LMP2B localise to perinuclear vesicles, the ER (Endoplasmic reticulum) and Golgi network indicating that LMP2 may localise to different regions in a cell type dependent manner (Lynch *et al* 2002; Dawson et *al*, 2001).

The cytosolic amino terminal tail of LMP2A contains 8 tyrosine (Y) residues, 2 of which (Y74 and Y85) form an immunoreceptor tyrosine-based activation motif (ITAM) (Fruehling *et al.*, 1996; Fruehling and Longnecker, 1997; Fruehling *et al.*, 1998; Longnecker, 2000). When phosphorylated the ITAM present in the B cell receptor (BCR), it plays a central role in mediating lymphocyte proliferation and differentiation by the recruitment and activation of the Src family of protein tyrosine kinases (PTKs) and the Syk PTK by sequestering these tyrosine kinases and blocking the translocation of the BCR into lipid rafts (Dykstra *et al.*, 2001). Thus, the LMP2A ITAM has been shown to be responsible for blocking BCR-stimulated calcium mobilisation, tyrosine phosphorylation and activation of the EBV lytic cycle in B cells (Miller *et al.*, 1995). Another tyrosine residue in the LMP2A amino terminal domain (Y112) is also required for efficient binding of *Src* family PTKs, particular Lyn (Longnecker *et al.*, 1991; Burkhardt *et al.*, 1992; Longnecker, 2000). LMP2A inhibits BCR signal transduction cascades initiated by cross-linking of surface immunoglobulin (sIg), CD19 or MHC class II molecules (Miller *et al.*, 1993; Miller *et al.*, 1995a). The N terminal tail also has multiple serine (Ser)/threonine (Thr) residues, which may bind Ser/Thr Kinases, and proline rich regions. It has already been confirmed that S15 and S102 are phosphorylated by MAPK *in vitro*, and MAPK was demonstrated to associate with LMP2A *in vitro* (Panousis and Rowe, 1997). LMP2B, lacking the receptor-like amino terminal domain of LMP2A, might downregulate the activity of LMP2A by increasing the spacing between the receptor-like tail domains of individual LMP2A molecules of the plasma membrane LMP2 aggregates (Longnecker, 2000).

The proline rich regions have been postulated to recruit proteins containing either Src Homology 3 (SH3) domains (Pawson and Gish, 1992) or WW domains (Sudol, 1996). SH3 domains are similar to SH2 domains in being non-catalytic regions that recruit kinases and adaptor proteins that are important in linking receptors to their signalling pathways. WW domains consist of paired tryptophan residues separated by 20-22 amino acids. LMP2A has been shown to contain two such regions in the amino terminal tail, which bind ubiquitin ligases (Ikeda *et al.*, 2000; Ikeda *et al.*, 2001). More specifically, it was demonstrated that members of the Nedd 4 ubiquitin ligase family associate with LMP2A (Winberg *et al.*, 2000; Ikeda *et al.*, 2003); this may decrease LMP2A activity. The structure and function of LMP2 is shown in Figure 1.3.

Figure 1-3 Overview of LMP2 structure and function

LMP2A and LMP2B share the C terminal region and the 12 transmembrane spanning domains (Laux *et al.*, 1989; Sample *et al*, 1989). LMP2A has an additional hydrophilic 119 amino acid N terminal domain (Longnecker, 2000). This N terminal region contains multiple tyrosine residues (Y) which can interact with Src family tyrosine kinases Syk and Lyn. The latter interaction sequesters Lyn from the BCR complex, resulting in inhibition of B-cell signalling pathways. The PI3-K and Akt pathways are also activated, leading to cell survival (Damania, 2004). LMP2A also recruits NEDD4-like ubiquitin protein ligases through phosphotyrosine (PY) motifs, and these promote the degradation of Lyn by an ubiquitin-dependent mechanism (Ikeda *et al.*, 2000). LMP2A interacts with the ERK1, MAPK and this results in the phosphorylation of two serine residues (Ser15 and Ser102) in LMP2A, and might contribute to LMP2A-induced activation of JUN (Chen *et al.*, 2002). No function has yet been ascribed to LMP2B; however the C terminal tail has been demonstrated to contain a clustering signal (Matskova *et al*, 2001).



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1.6.6.2 Function of LMP2A and LMP2B in B cells

LMP2A blocks normal B-cell signal transduction by mimicking an activated B-cell receptor (BCR) (Miller *et al.*, 1995, Portis *et al.*, 2002). This function is dependent upon the LMP2A cytoplasmic amino terminal domain and was subsequently shown to map to critical tyrosine residues within motifs that interact with Lyn and Syk (Longnecker *et al.*, 1991; Burkhardt *et al.*, 1992). LMP2 also provides a survival signal to primary B lymphocytes (Caldwell *et al.*, 1998; Portis and Longnecker, 2004; Fukuda and Longnecker, 2004). Expression of LMP2A in the B cells of transgenic mice abrogates normal B cell development allowing immunoglobulin-negative cells to colonise peripheral lymphoid organs suggesting that LMP2A can drive the proliferation and survival of B cells in the absence of signalling through the B cell receptor (BCR) (Caldwell *et al.*, 1998). Furthermore, bone marrow cells from LMP2A TgE transgenics in both wild-type and recombinase-activating gene (RAG)-deficient backgrounds form colonies in IL-7-containing methylcellulose, indicating that LMP2A bypasses the requirement for Ig rearrangement and allows for IL-7-driven B-cell proliferation (Caldwell *et al.*, 2000). The LMP2A-mediated effects on B-cell development and survival in vivo have also been shown to require the signalling component Syk, BLNK (SLP-65) and Bruton's tyrosine kinase (Btk) (Merchant *et al.*, 2000; Engels *et al.*, 2001; Merchant and Longnecker, 2001). LMP2A activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which normally provides a survival signal in response to BCR signalling (Scholle *et al.*, 2000; Swart *et al.*, 2000; Fukuda and Longnecker, 2004). A recent study showed that LMP2A mediates the survival of primary, BCR-negative B cells by constitutively activating the Ras/PI3K/Akt pathway (Portis and Longnecker, 2004; Fukuda and Longnecker, 2004). DNA

microarrays also demonstrate that expression of LMP2A in murine B cells interferes with global transcription factor regulation for proper B-cell development when expressed during B lymphopoiesis (Portis and Longnecker, 2003; Portis *et al.*, 2003). Taken together these data support a role for LMP2 in modifying the normal B cell development program to favour the maintenance of EBV latency in the bone marrow and to prevent inappropriate activation of the EBV lytic cycle.

LMP2A uses ubiquitination to regulate its own activity and might also utilize ubiquitination to modulate cellular signalling pathways, which regulate cell survival and differentiation (Ikeda *et al.*, 2003; Portis *et al.*, 2004). LMP2A signalling appears to be regulated in B cells by association of LMP2A with members of the HECT-domain containing Nedd4-family ubiquitin protein ligases, including AIP4/Itchy, WWP2, Nedd4 and KIAA0439/Nedd4-2 (Ikeda *et al.*, 2000; Winberg *et al.*, 2000). Nedd4-family members bind specifically to PY motifs present in the cytoplasmic domain of LMP2A, resulting in the ubiquitination and degradation of LMP2A and its associated proteins, such as the PTK Lyn (Winberg *et al.*, 2000; Ikeda *et al.*, 2001; Ikeda *et al.*, 2002). Although most proteins are specifically ubiquitinated at lysine residues, LMP2A is ubiquitinated at its N-terminal methionine residue (Ikeda *et al.*, 2002). This uncommon N-terminal ubiquitination is also observed in LMP1 (Aviel *et al.*, 2000), indicating that EBV might specifically utilize this type of host cell modification during viral latency. Experiments crossing LMP2A transgenic mice with mice deficient for Itchy, the mouse homologue of human AIP4, demonstrate that Itchy negatively regulates LMP2A function *in vivo* (Ikeda *et al.*, 2003). Interestingly, the extent of LMP2A expression and its emanating signal strength has been shown to influence B-cell differentiation during development. Low

LMP2A expression does not inhibit immunoglobulin (Ig) rearrangement, BCR expression or normal B-cell differentiation into follicular and marginal zone B cells (Casola *et al.*, 2004; Caldwell *et al.*, 2000). By contrast, high LMP2A expression inhibits BCR expression and results in exclusive B-1 differentiation in the bone marrow and peripheral lymphoid organs (Casola *et al.*, 2004; Ikeda *et al.*, 2004). It is therefore likely that LMP2A utilizes ubiquitin-mediated degradation through the proteasome complex to regulate the strength of its own signal, which might allow LMP2A to modulate B-cell processes, such as differentiation, activation or survival.

Several reports have demonstrated that neither LMP2A and nor LMP2B are essential for the immortalisation of B cells; they do not affect the ability of EBV to transform primary B lymphocytes into LCLs or LCL survival (Longnecker *et al.*, 1992; Kim and Yates, 1993; Longnecker *et al.*, 1993a; Longnecker *et al.*, 1993b; Speck *et al.*, 1999; Konishi *et al.*, 2001), but there is disagreement over the possible influence of LMP2 as an enhancer of transformation efficiency (Brielmeier *et al.*, 1996; Dawson *et al.*, 2001). LMP2A and LMP2B are controlled by two separate promoters and may be differentially expressed depending on the environment of the latently infected lymphocyte (Longnecker and Miller 1996). Thus, the differential expression may cause either lytic activation or maintenance of latent infection.

1.6.6.3 LMP2A and LMP2B in epithelial cells

NPC tumours show consistent expression of LMP2A at the mRNA level with variable levels of LMP1 expression. LMP2A was detectable in over 90% of NPC biopsies whereas LMP2B could only be detected in 39% (Brooks *et al.*, 1992; Busson *et al.*, 1992). Recently, the expression of LMP2A protein was reported in

50% of NPC biopsies by immunohistochemical staining (Heussinger *et al*, 2004). LMP2A can indirectly interact with LMP1 to enhance LMP1 signalling in epithelial cells, especially through the ITAM motif (Dawson *et al*, 2001). The half-life of LMP1 was almost doubled in the presence of LMP2A (Dawson *et al*, 2001). Another study demonstrated that LMP2A has other novel effects on epithelial cell signalling; the expression of LMP2A in HEK293 cells led to the phosphorylation of ERK and JNK, which are components of the MAPK signalling pathway (Chen *et al*, 2002). The phosphorylation of LMP2A in epithelial cells is triggered by cell adhesion, but this does not appear to be mediated via Src kinases (Scholle *et al*., 1999). It was speculated that this phosphorylation is due to C-terminal c-Src kinase (Csk, a negative regulator of Src kinases). Csk has been shown to regulate the activity of focal adhesion kinase (FAK) and paxillin, providing a mechanism whereby LMP2A may influence cell spreading (Tremblay *et al*., 1996). Whereas LMP2A and LMP2B are not essential for B cell transformation, LMP2A can transform epithelial cells and enhance their adhesion and motility; effects that might be mediated by the activation of the phosphatidylinositol-3-kinase-Akt pathway (Scholle *et al*., 2000). Recently, it was demonstrated that expression of LMP2A and LMP2B had no effect on the morphology of squamous epithelial cells in monolayer culture, but their expression was associated with an increased capacity to spread and migrate on extracellular matrix (Allen *et al.*, 2005). The use of selective pharmacological inhibitors has established a role for tyrosine kinases in this phenotype but ruled out contributions of PI3k, ERK/MAPK and protein kinase C. LMP2B may directly engage signalling pathways to influence epithelial cell behaviour such as cell adhesion and motility (Allen *et al.*, 2005). All the evidence points towards an

important role for LMP2A in NPC tumour development, but the mechanism is unclear.

Less than 10% of gastric carcinomas are EBV positive, and these tumours display a restricted pattern of EBV latent-gene expression which includes the EBERs, EBNA1, LMP2A, BARTs and BARF1, similar to that seen in NPC (Imai *et al.*, 1994; Burgess *et al*, 2002; Karim and Pallesen, 2003; Alipov *et al.*, 2005). The role of LMP2 in the pathogenesis of gastric carcinoma remains to be determined.

1.6.6.4 LMP2A and LMP2B in Hodgkin's lymphoma

Appoxmately 50% of HL contains EBV DNA and viral gene expression is limited to EBNA1, LMP1 and LMP2 (Thorley-Lawson, 2001; Khan and Coates, 1994; Oallesen *et al.*, 1994; Wu *et al.*, 1990). LMP2A transcripts and protein are consistently detected in all the EBV positive HL tumour cells (Murray *et al.*, 2000, Kieff and Rickinson, 2000). HRS cells are presumed to be derived from germinal centre B cells that have lost Ig expression and should have undergone apoptosis (Kuppers *et al.*, 2002; Marafioti *et al.*, 2000). A recent study using DNA microarrays reports that LMP2A induces a global down-regulation of B-cell-specific transcription factors and signalling molecules, many of which are also significantly down-regulated in HRS cells (Portis and Longnecker, 2003; Portis *et al.*, 2003). Although many studies have used LMP2 as a target for immunotherapy of HL (Rooney *et al.*, 1998; Dukers *et al.*, 2000; Bollarol *et al.*, 2004), the exact role of LMP2 in the pathogenesis of HL remains unknown.

1.6.7 EBERs

The EBV encoded small non-polyadenylated RNAs, EBERs1 and 2, which are166 and 172 nucleotides in length, respectively and the most abundantly expressed EBV RNAs in latently infected cells (Arrand and Rymo, 1982). The genes

encoding the EBERs are situated adjacent to one another in the right hand 1 kb of the *Bam*HI fragment. EBER1 is transcribed by RNA polymerase II and III, whereas EBER2 is transcribed by RNA polymerase III (Howe and Shu, 1989; Howe and She, 1993). While EBERs 1 and 2 share only 54% sequence similarity in their primary sequences, they are predicted to have similar secondary structures, displaying base paired structures containing a number of short-stem loops. Once transcribed, the EBERs localise to the nucleus where they are found in ribonucleoprotein (RNP) particles complexed to cellular protein La (Lerner *et al.*, 1981) and to EBER-associated protein (EAP), better known as the ribosomal protein L22 that has been implicated as a potential oncoprotein in another context (Toczyski and Steitz, 1991; Nucifora *et al.*, 1993; Toczyski *et al.*, 1994). The EBER sequences are highly conserved between types 1 and 2 EBV; this and their consistent expression in all known forms of EBV latency may be indicative of a critical role for these RNAs in EBV biology. However, EBER-knockout EBV recombinants are not impaired for primary B cell transformation (Swaminathan *et al.*, 1991). Furthermore, no differences were detected in the growth of the resulting LCLs or in the permissivity of these cells for lytic replication following induction.

1.6.8 Other latent transcripts

1.6.8.1 BamHI A transcripts

In addition to the latent antigens, a family of differentially spliced complementary-strand *Bam*HI A rightward transcripts (CSTs, BARTs or BARF0s) have been detected in BL cells, NPC tumours, HL tumours, latently infected lymphocytes from peripheral blood and primary infected B lymphocytes in culture (Gilligan *et al.*, 1990; Chen *et al.*, 1992a; Karran *et al.*, 1992; Brooks *et*

al., 1993; Chen *et al.*, 1999; Smith *et al.*, 2000; Deacon et al., 1993). Several putative ORFs have been identified in the *Bam*HI A family of transcripts which may encode proteins in latently infected cells; these include BARF0, encoded in the 3' ORF in exon 7; an extension of BARF0, RK-BARF0; RPMS1 encoded by an ORF spanning exon 4 and part of exon 5 of some *Bam*HI A transcripts; and A73 encoded by an ORF from components of exons 6 and 7 (Sadler and Raab-Teaub, 1995; Fries *et al.*, 1997; Kienzle *et al.*, 1999; Smith *et al.*, 2000). BARF1 is another transcript from the *Bam*HI A region, which encodes 31kDa protein. It has been detected in NPC and gastric carcinoma as a latent secreted protein (De Jesus *et al.*, 2003; Smith *et al.*, 2000), which can activate cell cycle as a growth factor (Sall *et al.*, 2004). Although the function and protein coding capacity of the BamHI A transcripts has not been fully elucidated, the finding that they are consistently expressed in all EBV-associated epithelial and B cell tumours and in tightly latent peripheral lymphocytes suggests they may have an important, as yet undiscovered, function in viral persistence. However, from recombinant virus experiments, the *Bam*HI A transcripts are not essential for transformation of primary B cells in vitro, LCL outgrowth or survival (Robertson *et al.*, 1994; Kempkes *et al.*, 1995).

1.6.8.2 BHRF1

The BHRF1 coding sequence is found at the 3' end of some Cp/Wp-initiated EBNA transcripts in latently infected cells. However, while the BHRF1 protein, a homologue of cellular Bcl2 may normally function to promote productive EBV infection, it might also contribute to the development of EBV-associated tumours (Dawson *et al.*, 1995). BHRF1 is not essential for the transformation of primary B lymphocytes, or for outgrowth or survival of LCLs, or for EBV replication

(Marchini *et al.*, 1991; Lee and Yates, 1992). The role of BHRF1 in EBV infected cells has yet to be elucidated but it has been confirmed as a functional Bcl-2 homologue and has antiapoptotic effects when expressed in EBV-negative B cell lines (Henderson *et al.*, 1993; Horner *et al.*, 1995). Its physiological role could be to maintain the viability of EBV-infected cells following entry into lytic cycle, thereby extending the time over which viral progeny can be produced.

1.7 Lytic cycle

There is no naturally permissive system in which to study replication. In EBV latently infected cells, only a small percentage of cells spontaneously enter lytic cycle, so it is not easy to analyse the EBV lytic cycle. Lytic cycle can be induced in latently infected cells by treatment with phorbol ester, calcium ionophore treatment, 5'-azacytidine, sodium butyrate, vector-mediated BZLF1 expression and sometimes by sIg cross-linking or superinfection with P3HR1 virus (Luka *et al.*, 1979; Zur Hausen and Fresen; 1978; Ben-Sasson and Klein, 1981). In the best-characterised system involving anti-Ig induction of the Latency I Akata-BL cell line, EBV lytic cycle takes 48-72 hours (Takada and Ono, 1989). The first proteins to be expressed are the products of the immediate early genes, BZLF1 and BRLF1, classified as such because their transcription post-induction is independent of protein synthesis (Flemington and Speck, 1990).

Some latency-associated proteins are expressed during lytic cycle. For example, EBNA1 continues to be expressed in lytic cycle but now transcription initiates from the lytic Fp promoter, located in the *Bam*HI F fragment (Lear *et al.*, 1992; Schaefer *et al.*, 1995a). The other EBNAs appear to be downregulated if lytic cycle is induced in a latency III background, possibly reflecting the reported ability of BZLF1 to repress Cp activity (Kenney *et al.*, 1989). In at least some, the

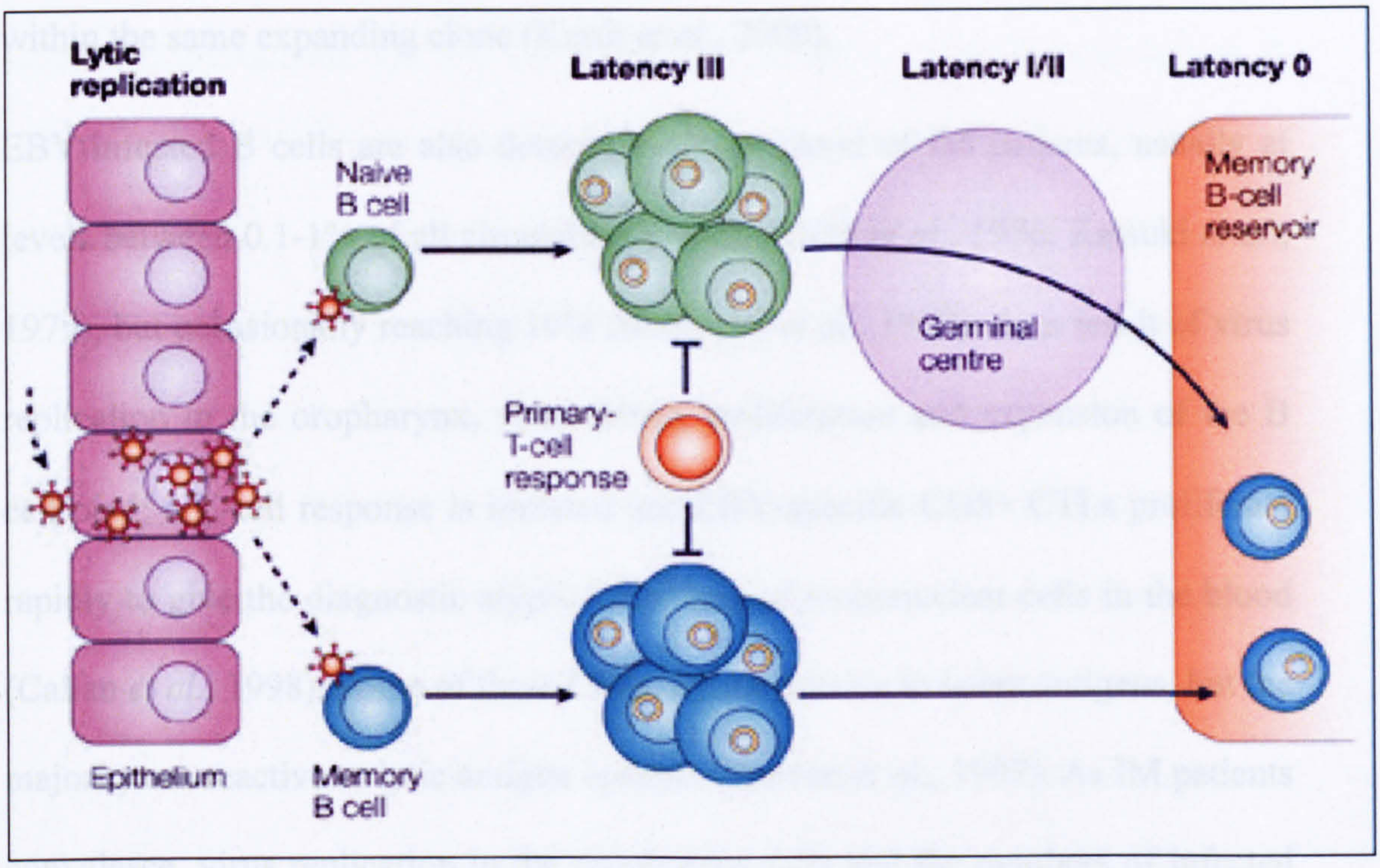
LMP promoters are also active in lytic cycle, with expression of LMP1 as an early lytic cycle protein (Rowe *et al.*, 1992).

1.8 Primary EBV infection in vivo and. viral persistence

EBV is naturally transmitted via the salivary route and infection results in life-long EBV persistence. Primary EBV infection may occur within cells of the oropharynx. It was initially believed that EBV infected the epithelial cells of the oropharynx and subsequently spread to infiltrating B lymphocytes, since EBV replication was detected within epithelial cells shed in the throat of IM patients (Sixbey *et al.*, 1984; Yao *et al.*, 1985). However, doubt was cast on this hypothesis when examination of IM tonsils in tissue sections failed to detect infected epithelial cells (Niedobitek *et al.*, 1997). Instead replicating virus could be found in B cells situated near epithelial crypts and it is now thought that initial EBV infection requires virus replication in B lymphocytes infiltrating the oropharynx (Tao *et al.*, 1996; Karajannis *et al.*, 1997). A model of EBV infection is shown in Figure 1.4.

Figure 1-4 A model of primary EBV infection

In the oropharynx, EBV infects naive B cells and expresses the full spectrum of latent proteins (latency III programme: EBNA1, LMPs 1 and 2A, EBNA3s and LP). The virus can thereby drive the activation and proliferation of these B cells, which then migrate to lymphoid follicles and form germinal centres. Many of these proliferating cells are removed by the emerging latent-antigen-specific primary-T-cell response. Concomitantly, expression of EBNA3s and LP are downregulated, leaving EBNA1 and the LMPs expressed (latency II programme), some which establish a stable reservoir of resting viral-genome-positive memory B cells, in which viral antigen expression is mostly suppressed (latency 0), but to evade a host immune response. An alternative view therefore envisages infection of pre-existing memory cells as a direct route into memory B cell.



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Analysis showed that EBV-infected tonsillar B cells are heterogeneous both in morphology and viral antigen expression in IM (Niedobitek *et al.*, 1997; Kurth *et al.*, 2003). While there is clearly a proliferating compartment of LCL-like cells expressing both EBNA2 and LMP1, there are also smaller cells expressing EBNA2 by staining and a number of very large cells with Hodgkin's lymphoma-like morphology that are EBNA2-negative and LMP1 positive. Cells with these different morphologies and apparently different latency patterns can even exist within the same expanding clone (Kurth *et al.*, 2000).

EBV-infected B cells are also detectable in the blood of IM patients, usually at levels between 0.1-1% of all circulating B cells (Klein *et al.*, 1976; Katsuki *et al.*, 1979), but occasionally reaching 10% (Robinson *et al.*, 1980). As a result of virus replication in the oropharynx, virus-driven proliferation and expansion of the B cell pool; a T cell response is initiated and EBV-specific CD8+ CTLs proliferate rapidly to give the diagnostic atypical numbers of mononuclear cells in the blood (Callan *et al.*, 1998). Some of these CTLs are responsive to latent antigens, but the majority are reactive to lytic antigen epitopes (Steven *et al.*, 1997). As IM patients convalesce, virus replication in the oropharynx falls and the numbers of infected peripheral B cells reduces. However, despite the strong CTL response the virus establishes a lifelong persistence and remains detectable at very low levels both as infectious virus in throat washings and as a latent infection of the B cell pool (Yao *et al.*, 1985). The relationship between these two sites of virus persistence remains ill-defined and, while the balance of evidence favours the B cell pool as the essential reservoir of EBV persistence, there remains the possibility of a separate oropharyngeal reservoir of persistence, perhaps involving chronic replication

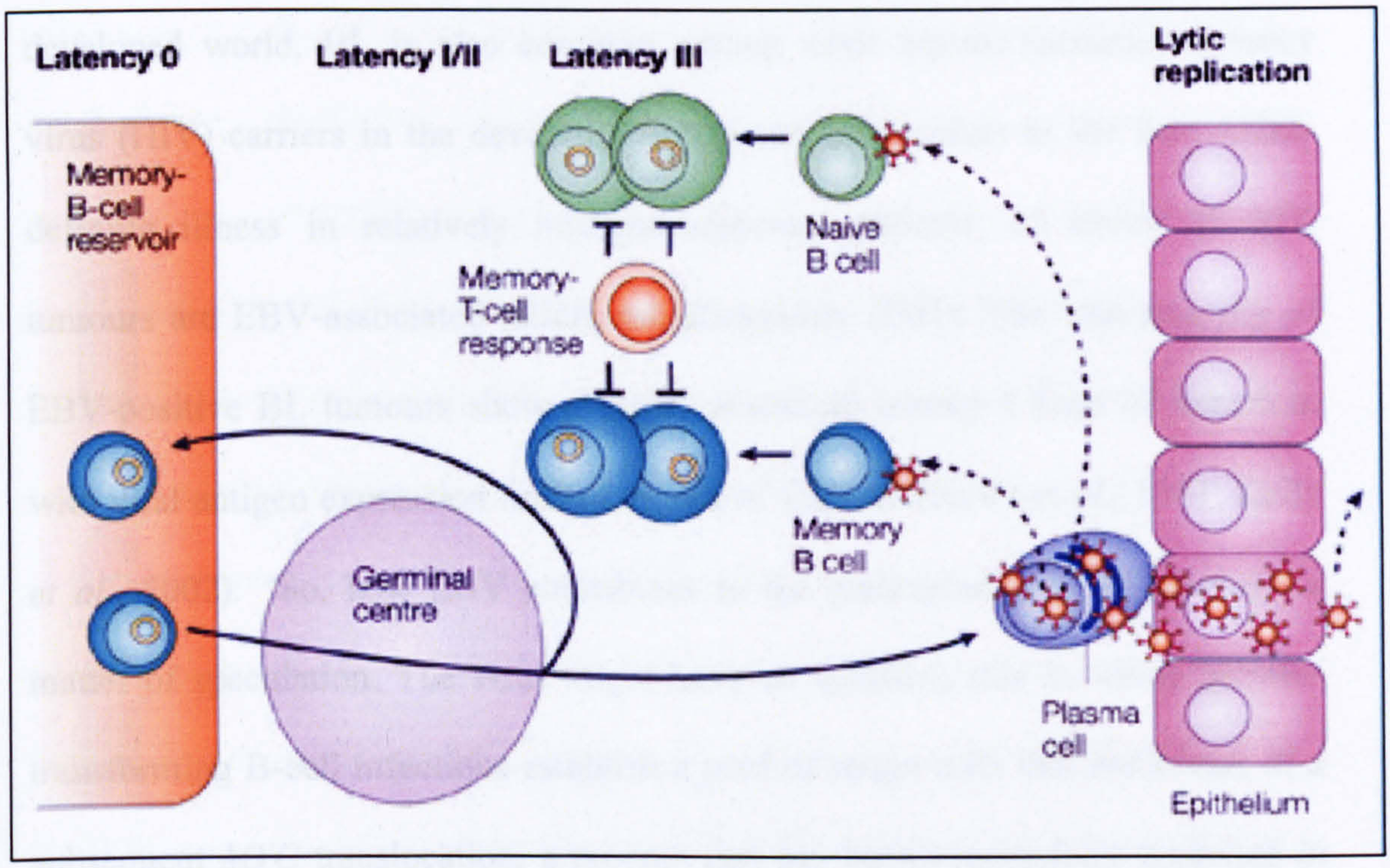
rather than true latency (Sixbey *et al.*, 1984). A model of EBV persistence is shown in Figure 1.5.

Evidence favouring B cells as the site of EBV persistence includes i) total body irradiation destroying the lymphoid system but leaving epithelium intact, can clear EBV from a virus carrier (Gratama *et al.*, 1988); ii) treating patients with acyclovir eradicated EBV replication in the oropharynx but did not affect EBV residing in the peripheral blood (Yao *et al.*, 1989); and iii) patients with X-linked agammaglobulinaemia (XLA), and therefore incapable of producing mature B cells remain EBV negative (Faulkner *et al.*, 1999). In the blood the site of EBV persistence had now been identified as the $CD19^+$, $CD23^-$, $CD80^-$ ($B7^-$), $CD27^-$ resting memory B cell population (Miyashita *et al.*, 1997; Babcock *et al.*, 1998; Malbran *et al.*, 2004), but how EBV preferentially colonises this cell compartment is widely debated. Downregulation of EBV latent gene expression from the initial Latency III infection is a crucial step in viral persistence in the immunocompetent host and so another possibility is that both naïve and memory B cell are susceptible to Latency III infection but only pre-existing memory cells have the capacity to switch latency type in vivo; therefore only these cells can survive in the immunocompetent host. RT-PCR analyses of latent gene transcripts expressed in peripheral B cells isolated from healthy virus carries have detected EBER RNAs, *Bam*HI A transcripts, LMP2A transcripts and trace levels of Qp-initiated EBNA1 transcripts (Qu and Rowe, 1992; Tierney *et al.*, 1994; Miyashita *et al.*, 1997; Chen *et al.*, 1999). However, another report showed the major infected population in the blood of immunosuppressed patients may not express the known latent proteins (Babcock *et al.*, 1999).

EBV can presumably be reactivated from memory B cells into lytic cycle in vivo (as well as in vitro immediately following the culture of circulating B cells from virus carriers). And this reactivation may serve to establish new transformation events that replenish the infected B cell pool (Babcock and Thorley-Lawson, 2000). Infection by EBV is controlled by both cellular and humoral immune mechanisms (Hochberg *et al.*, 2004a; Hochberg *et al.*, 2004b). However, the immune system is unable to eliminate the virus completely, and as a consequence, viral shedding and virus-infected cells persist at a low level in memory B cells (Laichalk *et al.*, 2002).

Figure 1-5 A model of EBV persistence

As B cells recirculate to the oropharynx, a switch into the EBV lytic cycle might occur, possibly triggered by maturation of B cells into plasma cells, allowing for virus replication, shedding into saliva and transmission both to new hosts and to previously uninfected B cells within the same host. This might initiate new growth-transforming latency III infections of naive and/or memory B cells and might possibly replenish the B-cell reservoir, but are more likely to be efficiently removed by the now well-established memory-T-cell response.



Taken from Young and Rickinson (2004) Nature Review

1.9 EBV associated malignancies

1.9.1 Burkitt's lymphoma (BL)

EBV is present in all cases of 'endemic' Burkitt's lymphoma (BL)-the high-incidence form of the tumour that affects children in areas of Africa and New Guinea in which malaria is holoendemic. EBV is also present in up to 85% of cases in areas of intermediate incidence such as Brazil and North Africa, but in only 15% of the low-incidence 'sporadic' tumours that are seen in children in the developed world. BL is also common among adult human immunodeficiency virus (HIV) carriers in the developed world and often arises as the first AIDS-defining illness in relatively immunocompetent patients, of which 30–40% tumours are EBV-associated (Kieff and Rickinson, 2001). The vast majority of EBV-positive BL tumours show a highly restricted latency I form of infection, with viral antigen expression limited to that of EBNA1 (Rowe *et al.*, 1987; Kelly *et al.*, 2002). So, how EBV contributes to the pathogenesis of BL remains a matter of speculation. The virus might have an initiating role in which growth-transforming B-cell infections establish a pool of target cells that are at risk of a subsequent *MYC* translocation, a process that has been successfully modelled *in vitro* (Polack *et al.*, 1996). EBV might contribute to the BL phenotype through the latency-I-associated genes themselves. *EBNA1* is an candidate, but report about its oncogenicity remains controversial (Wilson, 1996), and its contribution to virus-induced B-cell transformation *in vitro* might be limited to maintenance of the viral genome (Humme *et al.*, 2003; Kang *et al.*, 2001); however, another recent report showed EBNA1 promoted cell survival (Kennedy, 2003). Irrespective of their EBV status, the phenotype of BL cells is similar to that of germinal centroblasts, and the detection of ongoing Ig-gene mutation in tumour cells supports the

suggestion that they originate in germinal centres (Chapman 1995; Harris *et al.*, 2001).

1.9.2 Hodgkin's lymphoma (HL)

Hodgkin's lymphoma (HL) is an unusual tumour, characterized by atypical, large tumour cells known as Hodgkin (mononucleated) and Reed–Sternberg (multinucleated) (HRS) cells (Weiss, 2000). HRS cells usually represent less than 1% of the cells in the HL tumour tissue, the vast majority of cells are non-malignant T cells, B cells, macrophages, eosinophils, plasma cells and others (Weiss *et al.*, 2000). The incidence of HL in the Western world is about 2.5–3 new cases per 100,000 people per year and it is more common among people 15 to 35 and 50 to 70 years old (Glaser *et al*, 1997). In EBV positive HL, young patients under 15 years have better prognosis than older patients with subtype NS (nodular sclerosis) (Keegan *et al.*, 2005). HL is has a high cure rate, about 90% of patients achieve a long-term remission following treatment.

In 1832 Thomas Hodgkin first described the case of lymphoid lesion that was later named Hodgkin's disease (HD), and in 1902 and 1898, Dorothy Reed and Carl Sternberg described a peculiar cell type in HD tissues which was referred to as the Reed-Sternberg (RS) cell (Hodgkin, 1832; Reed, 1902; Sternberg, 1898). Based on the differences in the cellular composition and the histology of lesions, HL is divided into lymphocyte predominance and classical Hodgkin lymphoma (LPHL and cHL). In LPHL, the Hodgkin cells are also called L&H cells (lymphocytic and histocytic). cHL is further distinguished into four subsets of classical HL, nodular sclerosis (NS), mixed cellularity (MC), lymphocyte depletion (LD), lymphocyte rich (LR). NS and MC are the most common types, which together comprise 80% to 90% of all HL. NS is more common in adults in

their 20's and 30's, particularly females. HRS cells of HL are large cells that can be more than 50μm in diameter and have a moderate amount of cytoplasm and a clearly visible nucleolus in each nucleus (Weiss, 2000).

The origin of these cells was finally clarified by using microdissection to isolate single HRS cells from frozen tissue sections following by analysis of the rearranged immunoglobulin (Ig) genes. In nearly all the cases, the HRS cells carried a rearranged Ig heavy and light chain gene, and by this these cells were identified as B cells (Brauninger *et al.*, 1999; Kuppers *et al.*, 1994). Moreover, by the detection of the identical Ig V gene rearrangements in the HRS cells of a given case, their monoclonality was demonstrated (Kuppers *et al.*, 1994; Marafioti *et al.*, 2000). It was reported recently that all cases of HL with crippling mutations that prevent BCR expression are EBV positive (Brauninger *et al.*, 2006).

Approximately half of HL cases have EBV DNA within HRS cells and express at least four viral products, the so-called latency II programme (Deacon *et al.*, 1993): EBERs, EBNA1, LMP1, LMP2 and B*am*HI A transcripts. Type-1 EBV has been detected in most HL tissues and type-2 virus is found in a small proportion of cases. The presence of EBV in HL depends on a number of factors, including the patent's country of residence, histological subtype, sex, ethnicity and age. EBV-positive HL is less common than EBV-negative HL in well-developed populations compared with poorly developed communities (Glaser *et al.*, 1997). EBV is more commonly associated with the mixed-cellularity subtype of HL and less frequently with the other forms of this disease (Glaser et al., 1997). The association of EBV with HL has been intensely investigated over the last few years; however, the contribution of EBV to the pathogenesis of HL remains unknown. A recent report showed that EBV infection in HL cells induces the

expression of autotoxin (a secreted tumour-associated factor) and proliferates HL cells; this indicates that EBV infection has a contribution to the pathogenesis of HL. Furthermore, in addition to the role of EBV in the aetiology of HL, the function of EBV-latent antigens, such as LMP1 and LMP2, in the malignant HL cells requires investigation.

The expression pattern of EBV-encoded genes in HRS cells indicates a role for EBV in the rescue and transformation of pre-apoptotic germinal-centre B cells (Kuppers and Hansmann, 2005). Two of the main survival signals for germinal-centre B cells are mediated through the BCR and through stimulation of CD40 (Liu *et al.*, 1989). These signals can presumably be replaced by LMP2A and LMP1, respectively, both of which are expressed in the EBV-positive HRS cells (Herbst *et al.*, 1991; Niedobitek *et al.*, 1997; Pallesen *et al.*, 1991). Therefore, it might be the expression of LMP1 and LMP2A in the EBV-infected germinal-centre B cells that allows them to survive despite the presence of crippling mutations (Kuppers and Hansmann, 2005; Brauninger *et al.*, 2006).

Recently it was reported that LMP2A might contribute significantly to the HL phenotype by altering cellular gene transcription; these included downregulation of the specific gene expression for B cell development (Küppers *et al.*, 2002, Portis *et al.*, 2003; Portis and Longnecker, 2003). The ability of LMP2A to substitute for a functional BCR was clearly demonstrated in EBV infected B cells, even if in cells that failed to hypermutate their BCR successfully (Casola *et al.*, 2004). Therefore, the rescue of pre-apoptotic germinal centre B cells by LMP2A might be a crucial step in their transformation. However, LMP2A might have few, if any, functions in the established HRS tumour clone: It was reported that HRS cells have lost the expression of most B-cell-specific genes, including the kinases

Syk and Lyn and the adaptor molecule B-cell linker (BLNK, also known as SLP65) (Schwering *et al.*, 2003). These molecules are not only crucial for BCR signalling, but are also essential components of the signalling pathway used by LMP2A (Engels *et al.*, 2001). Therefore, LMP2A might not be able to mimic the presence of a BCR in HRS cells, at least through the known signalling pathways.

1.9.3 Post transplant lymphoproliferative disease (PTLD)

Immunosupressed transplant patients have been shown to be at increased risk of developing a particular spectrum of EBV-associated B lymphoproliferative lesions/lymphomas termed PTLD (Penn, 2000). The disease normally arises within the first two years after transplantation and presents as multifocal lesions within the lymphoid tissues, the central nervous system (CNS), the liver or the transplanted organ (Nalesnik *et al.*, 1988). There is a high association with EBV (Nalesnik, *et al.*, 1988. Weiss and Movahed, 1989; Nalesnik, 1998). Patients EBV-negative at the time of transplantation are at a 20-fold increased risk of developing the disease, since they frequently suffer primary infection post-transplant and have no pre-existing EBV immunity upon which to call (Ho *et al.*, 1985). Measuring and monitoring increased EBV loads in the PBMCs of transplant patients may serve as a useful diagnostic tool to predict the likelihood of PTLD onset (Rowe *et al.*, 1997; Baldanti *et al.*, 2000). Most early onset PTLD tumours display a type III latency pattern of EBV gene expression, with detectable EBNA1, EBNA2 and LMP1 expression, consistent with in vitro LCL outgrowth in the absence of CTL responses (Young *et al.*, 1989; Rea *et al.*, 1994; Delecluse *et al.*, 1995). Interestingly, even within these largely LCL-like PTLD lesions, antibody staining reveals subpopulations of EBNA2-positive LMP1-negative and EBNA2 negative LMP1-positive cells; analogous to the

heterogeneity seen in EBV-expanding cell lines in IM tonsils. The full significance of this heterogeneity remains to be determined. However, other PTLDs clearly show a more restricted pattern of gene expression, with only EBNA1 protein detectable (Delecluse *et al.*, 1995; Oudejans *et al.*, 1995).

1.9.4 Nasopharyngeal carcinoma (NPC)

The EBV-associated, undifferentiated form of nasopharyngeal carcinoma (NPC) shows the most consistent worldwide association with EBV and is particularly common in areas of China and south-east Asia, reaching a peak incidence of around 20–30 cases per 100,000 (Yu and Yuan, 2002). However, NPC is uncommon disease in the west; where the incidence is less than 1 per 100,000 among Caucasians from North American and other Western countries. Incidence rates are high in individuals of Chinese descent, irrespective of where they live, and particularly in Cantonese males. In addition to this genetic predisposition, environmental cofactors such as dietary components (for example, salted fish) are thought to be important in the aetiology of NPC (Yu *et al.*, 1986). NPC tumours are characterized by the presence of undifferentiated carcinoma cells and a prominent lymphocytic infiltrate, and an interaction between tumour cells and lymphocytes seems to be crucial for the continued propagation of the malignant component.

EBV latent-gene expression in NPC is predominantly restricted to EBNA1 LMP2A, LMP2B, EBERs and the *Bam*HIA transcripts, with ~20% of tumours also expressing the oncogenic LMP1 protein (Raab-Traub, 2002). Southern-blot hybridization of DNA from NPC tissues demonstrates the monoclonality of the resident viral genomes, indicating that EBV infection takes place before the clonal expansion of the population of malignant cells (Raab-Traub and Flynn, 1986).

Studies of normal nasopharyngeal tissue and pre-malignant biopsies indicate that genetic events occur early in the pathogenesis of NPC, and that these might predispose to subsequent EBV infection. Higher frequencies of deletions on chromosomes 3p and 9p are found in histologically normal nasoharyngeal epithelia from southern Chinese, as well as in low- and high-grade dysplastic lesions (Chan *et al.*, 2000; Chan *et al.*, 2002). Extensive serological screening has identified increased EBV-specific antibody titres in high-incidence areas; in particular, IgA antibodies to the EBV capsid antigen and early antigens have proved useful in diagnosis and in monitoring the effectiveness of therapy (Zeng, 1985). More recent studies using real-time quantitative PCR to measure circulating tumour-derived EBV DNA in the blood of patients with NPC have shown that the level of pre-treatment EBV DNA is strongly associated with overall survival, and that post-treatment EBV DNA levels predict progression-free and overall survival (Chan, 2002). Association of EBV with the other more differentiated forms of NPC (WHO types I and II) has been shown, particularly in those geographical regions with a high incidence of undifferentiated NPC (Pathmanathan *et al.*, 1995). However, the precise role of virus infection in carcinogenesis and the contribution of epithelial cell infection to EBV persistence and replication remain unknown.

1.9.5 Gastric carcinoma

EBV is also found in ~10% of typical gastric adenocarcinomas, accounting for up to 75,000 new cases per year (Shibata and Weiss, 1992; Burgess *et al*, 2002; Tokunaga *et al.*, 1993). These tumours display a restricted pattern of EBV latent-gene expression (expression of EBERs, EBNA1, LMP2A, BARTs and BARF1), similar to that seen in NPC (Imai *et al.*, 1994). There is significant geographical

variation in the association of EBV with gastric carcinoma, which might be due to ethnic and genetic differences. EBV-positive gastric carcinomas have distinct phenotypic and clinical characteristics compared with EBV-negative tumours, including loss of expression of INK4A (also known as p16) and improved patient survival (Schneider *et al.*, 2000; Lee *et al.*, 2004). As in NPC, the precise role of EBV in the pathogenesis of gastric carcinoma remains to be determined, but the absence of EBV infection in pre-malignant gastric lesions supports the suggestion that viral infection is a relatively late event in gastric carcinogenesis (Zur Hausen *et al.*, 2004).

1.9.6 Virus-associated T-cell and NK-cell lymphomas

EBV has been linked to a proportion of peripheral T cell non-Hodgkin's lymphomas, in which 5-50% of the tumour cells are EBV detectable. The EBV infection was documented occurring subsequent to tumour development. This suggests that EBV infection might provide an additional growth/survival signal to the transformed T cells (Anagnostopoulos *et al.*, 1992; Anagnostopoulos *et al.*, 1995; Ho *et al.*, 1998; Niedobitec *et al.*, 1997; Tao *et al.*, 1995). Nasal or nasal-type NK-cell lymphoma presents a distinctive clinicophathologic entity, almost always associated with EBV. These NK-cell lymphomas, more common in Asia and especially in China (Jaffe *et al.*, 1996), characteristically present as a nasal destructive disease. The nasal lymphoma tissues display a latency II pattern of EBV gene expression characterized by expression of EBNA-1, LMP-1 and LMP-2A, but not EBNA-2 and LMP-2B (Chiang *et al*, 1996). EBV is B-lymphotropic, but how the virus accesses T cell lineages *in vivo* is still remain unknown. Most evidence indicates that such infections are rare, but confer a high risk of lymphoma development.

1.10 Aims and objectives

The contribution of LMP2 to the pathogenesis of virus-associated tumours is poorly understood. Therefore, this project seeks to address the contribution of LMP2A and LMP2B to the phenotype of HL and epithelial tumours.

The first objective of this work is to develop an *in vitro* system for the stable expression of LMP2 in an EBV-negative HL cells. Such cells will be used to examine the influence of LMP2 in cellular survival and proliferation and then to study the impact of LMP2A and LMP2B on cellular gene expression.

Finally, HEK293 cells will be used to generate cell lines expressing His-tagged LMP2A and LMP2B proteins. These will then be subjective to a novel methodology, stable isotope labelling amino acid in cell culture for the detection of LMP2A and LMP2B-interacting proteins.

CHAPTER TWO: MATERIALS AND METHODS

2 Materials and methods

2.1 Cell culture

2.1.1 Maintenance of cells

2.1.1.1 Hodgkin's lymphoma cells

KMH2, L428, L591 and L591-SD3 are Hodgkin's lymphoma derived cell lines (American Type Culture Collection, Rockville, MD). KMH2 was established from the pleural effusion of a patient with mixed cellularity HL (Kamesaki, *et al.*, 1986). L428 was derived from a pleural effusion that was histologically confirmed as HL (Schaadt *et al.*, 1980). L591 is a cell line established from pleural effusion of a female patient with histologically confirmed nodular sclerosis HL (Diehl *et al.*, 1982). L591-SD3 is a EBV-negative HL cell line generated by a series dilution of L591 (Baumforth *et al.*, 2005). All cell lines were maintained in exponential growth at 37°C, 5% CO_2 in 75 cm^2 flasks in RPMI 1640 medium (Gibco) supplemented with 10% v/v selected B cell serum*, 2mM glutamine (Gibco) and 1% penicillin and strepmycin (Sigma). KMH2 and L428 cells stably infected with EBV or stably expressing LMP2 were maintained under 1mg/ml G418 selection.

* B cell Serum: a number of batches of Foetal Calf Serum (FCS) were obtained from several sources and tested for their ability to support B cell growth as measured by the efficiency of colony formation when plated out at clonal density. The batch that gave the most efficient colony formation was selected as the FCS for use in all B culture. FCS was sterilised by pressure filtration, tested for virus/mycoplasma and stored in 20ml aliquots at –20°C.

2.1.1.2 HEK 293 cells

HEK 293 cell line is an immortalised cell line derived from primary human embryonal kidney cells transformed by sheared human adenovirus type 5 DNA (Graham *et al.*, 1977). 293 cells were grown at 37°C, 5% CO_2 in 75 cm^2 flasks in DME/HEPES medium (Gibco) supplemented with 10% v/v FCS, 2mM glutamine (Gibco) and 1% penicillin and streptomycin (Sigma). Cells were trypsinised with 1% w/v trypsin in diaminothanetetra-acetic acid disodium salt (EDTA) (Gibco) at 37°C for 5 minutes, pelleted by centrifugation at 1500rpm for 5 minutes and resuspended at an appropriate concentration in complete DME/HEPES medium as above. 293 cells stably expressing LMP2 were maintained under 400ug/ml Zeocin (Invitrogen) selection.

2.1.2 Cryopreservation of cells

Typically, aliquots of 5-10 x 10^6 cells were pelleted by centrifugation at 1500rpm for 5 minutes and resuspended in 1ml freezing mix (50% v/v complete medium, 40% v/v FCS and 10% v/v dimethyl sulphoxide (DMSO). Cells were transferred to a cryopreservation tube in a freezing box (container surrounded by sponge soaked in isopropanol) and stored at -70°C for short-term storage, before being transferred to the vapour phase of a –180°C liquid nitrogen freezer for long-term storage.

2.1.3 Recovery of cells from liquid nitrogen

The cells were thawed quickly at 37°C to minimise to DMSO damage and transferred to a 15 ml centrifuge tube. Corresponding pre-warmed medium at 37°C was added to the cells dropwise to dilute out the DMSO and allow the cells to recover slowly. Cells were pelleted by centrifugation at 1500rpm for 5 minutes,

resuspended in corresponding cell medium and grown in a 25cm^3 flask at 37°C, 5% CO_2.

2.2 Infection of KMH2 and L428 cells with retrovirus carrying LMP2A, LMP2B and Neomycin resistance cassette

2.2.1 Generation of Retrovirus carrying LMP2A, LMP2B and Neo

A13 packaging cells were stably transfected with the pLNSX-LMP2A, pLNSX-LMP2B plasmids or empty vector pLNSX (Neo) (a kind gift from Dr. CW. Dawson, CRUK Institute, University of Birmingham). These stably transfected packaging cells were seeded into 6cm petri dishes and the recombinant retroviruses produced by these cells were shed into the surrounding media. The conditioned media containing retrovirus pLNSX-LMP2A, pLNSX-LMP2B or pLNSX only were collected after 48 hours incubation, which were then used to transduce KMH2 and L428 cells as described below.

2.2.2 Infection of KMH2 and L428 cells with retrovirus carrying LMP2A, LMP2B and Neomycin resistance cassette

Cells were counted and their viability was checked. The cells were pelleted and resuspended in fresh medium ($5x10^4$ cells/ml). 1 ml of each cell suspension was put into 6 well plates. 2ml of supernatant with retrovirus carrying LMP2A, LMP2B or Neomycin resistance gene were added to each corresponding well. 4µl polybrene (Sigma, 10µg/ml) was added to each well and mixed. 2ml medium was topped up to each well after the plates were incubated for 4 hours at 37°C. 48 hours post-infection, the growth medium was changed to medium containing G418 (1mg/ml, Sigma). After 10 days of drug selection, drug-resistant polyclonal populations were screened for expression of LMP2A and LMP2B by RT-PCR and

immunofluorescence staining. Drug-resistant, polyclonal populations were then expanded in the presence of G418 (1mg/ml).

2.3 DNA/RNA detection

2.3.1 Reverse Transcription (RT)

2.3.1.1 RNA extraction

Total RNA was extracted from cell lines (1-5x10^6 cells) using the Nucleospin® RNA II kit (MACHERY-NAGEL), following the instructions provided by the manufacturer. Cell suspension was centrifuged at 1500rpm for 5 minutes and supernatant was removed from the pellets. Cells were lysed in a solution containing beta-mercaptoethanol, which enables adsorption of RNA to a silica membrane and inactivates any RNase enzymes. The lysate was then loaded onto a silica membrane. Any trace of DNA was removed by adding extra DNAse solution. Membrane was washed using buffers provided from the kit to remove any contaminants such as salts, metabolites and cellular components. RNA was eluted in RNase-free water in a volume of 60µl to ensure maximal yield. The yield and quality of each RNA sample was determined using an UV-spectrophotometer assuming 1 A_{260} unit=40µg RNA.

2.3.1.2 cDNA reaction

LMP2-specific cDNA was made using AMV kit (Roche) and LMP2 reverse oligonucleotide primer (see Appendix 8.1.1). 200ng of total RNA was put into a 0.5 ml microfuge tube to perform the reverse transcription in a final 20µl volume containing 4 µl 5xRT buffer (50mM KCl, 10mM Tris-HCl, pH 8.3), 5.5 mM $MgCl_2$, 0.5 mM each of dATP, dCTP, dGTP and dTTP, 2.0 µM LMP2A

oligonucleotide reverse primer, 1 unit RNase inhibitor and 20 units reverse transcriptase. These cDNA synthesis reactions were incubated at 42°C for 1 hour, followed by heating at 95°C for 5 minutes to inactivate the reverse transcriptase enzyme. Positive and negative controls were included in each run.

2.3.1.3 LMP2 PCR

Red Hot DNA polymerase kit (ABgene) was used for conventional PCR. 2µl gene specific primed cDNA was aliquoted into 0.5 ml microfuge tubes and a reaction mix was added to give a final reaction concentration of 1x reaction buffer (50mM KCl, 10mM Tris-HCl, pH 8.3), 5.5 mM $MgCl_2$, 0.25 mM each of dATP, dCTP, dGTP and dTTP, 1.0 µM of each forward and reverse LMP2A oligonucleotide primers (See Appendix 8.1.1) and 5 units of Red Hot DNA polymerase in a total reaction volume of 50ul. Samples were placed in a PCR machine, heated to 94°C for 5 minutes, then subjected to 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute and extension at 72°C for 30 seconds with a final extension phase at 72°C for 10 minutes. LMP2-positive cell line (X-50-7) was used as positive control.

2.3.1.4 Semi-quantitiative PCR

1µg of RNA from each sample was used to make cDNA in 20 µl volume. PCR was run as described above with 2µl cDNA for 20, 25, 30 and 35 cycles. Annealing temperature was determined for each set of primers. 5µl of each PCR product was run on 1.2% Argarose gels. At the same time PCR for GAPDH was run alone side as a loading control.

2.3.1.5 Electrophoresis

The 10 µl PCR reactions were mixed with 5x gel loading buffer (0.25%w/v xylene cyanol, 0.25% w/v bromophenol blue, 30%w/v glycerol) and subjected to electrophoresis (110 volts) through 1.2% agarose gel containing 0.5 µg/ml ethidium bromide in 1xTBE running buffer (90mM Tris-borate, 2 mM EDTA). A 1 kbp DNA ladder (Roche) was also loaded alongside the samples so that the size of the PCR products could be determined. After electrophoresis, the PCR products, as stained by the ethidium bromide, were visualised with UV light on a transilluminator and Polaroid photographs taken.

2.4 Protein detection and analysis

2.4.1 Immunofluorescence Staining (IF)

2.4.1.1 Two-Step immunofluorescence

Size 12 Teflon-coated slides (Hendley-Essex) were autoclaved, 50 µl of fibronectin (Sigma, 10µg/ml in PBS) was added to each well; then slides were kept in a sterile petridish at 4°C overnight. On the next day, the fibronectin was washed off carefully with PBS 2-3 times for 5 minutes. Cell suspension with approximately $5x10^4$ to $1x10^5$ cells in 50ul of medium was loaded to each well and incubated at 37°C overnight. Slides were fixed with 4% PFA (Paraformaldehyde) for 10 minutes, washed with PBS twice for 5 minutes and permeabilized with 0.1% (vol/vol) TritonX-100 for 2 minutes. Antibodies were diluted to the appropriate concentration in 20% HINGS. Primary and secondary antibody incubations were 1h each, and the slides were washed twice with PBS after each incubation. The slides were covered with DEBCO (2.5 mg in 90 ml DEBCO and 10 ml PBS) and coverslips added. Slides were imaged by UV

microscopy. The primary and secondary antibodies were used as described in Table 2.1 and Table 2.2.

Table 2-1 Primary antibodies used for IF

Primary Ab	Specificity	Nature	Dilution	Supplier
Bala serum	LMP2	EBV-positive human serum	1:50	NPC patient
Anti-His Ab	His epitope	Mouse monoclonal	1:100	Upstate
Anti-IFI27 Ab	IFI27 epitope	Mouse monoclonal	1:100	Abcam
Anti-AICDA Ab	AICDA epitope	Rat monoclonal	1:100	Gift from Professor Niedobitek (Germany)

Table 2-2 Secondary antibodies used for IF

Secondary Ab	Conjugate	Dilution	Supplier
Goat anti-human IgG	FITC	1:200	Sigma
Goat anti-mouse IgG	AlexaFluor 594	1:200	Molecular Probes
Goat anti- mouse IgG	AlexaFluor 488	1:200	Molecular Probes
Goat anti-Rat IgG	AlexaFluor 488	1:200	Molecular Probes
Goat anti- Rat IgG	AlexaFluor 594	1:200	Molecular Probes

2.4.1.2 Dual Staining

For dual staining with two separate antibodies, the primary antibodies were derived from different animals and the secondary antibody to each was conjugated to a different fluorescent marker, e.g. one FITC or AlexaFluor 488 and the other TRITC or AlexaFluor 594. At each stage, both antibodies were diluted together in 20% HINGS to the appropriate concentration. The subsequent steps are the same as described above. Staining was viewed under two filters by UV microscope.

2.4.2 Western blotting

Cells were washed with PBS, pelleted, and then lysed with the cell lysis buffer (see Appendix 8.2.3) on ice for 30 minutes. The cell lysate was spun down at 13,000 rpm for 10 minutes at 4°C. Proteins were quantified using the BioRad DC protein Assay Kit (500-0120 BioRad, UK). The GSB gel sample buffer (50mM Tris pH6.8, 100mM dithiothreitol, 2% SDS, 10% glycerol, 0.05% bromophenol blue) was added to the protein samples and these were boiled for 5 minutes. Soluble proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to BioTrace NT membrane (VWR internatonal, UK) for immunoblotting. The membrane was blocked with 5% milk PBS-Tween buffer for 1 hour; then incubated overnight at 4°C with primary antibody. The membrane was washed for 5 minutes twice with PBST buffer and incubated for 1 hour with a 1:2000 dilution of HRP-conjugated secondary antibody. Specific antibody-protein complexes were detected using the Enhanced Chemiluminescence (ECL) kit (RPN2106; Amersham Pharmacia Biotech, UK). The primary and secondary antibodies used are listed see Table 2.3 and Table 2.4.

Table 2-3 Primary antibodies were used for western blotting

Primary Ab	Specificity	Nature	Dilution	Supplier
B-Actin Ab	Actin	Mouse monoclonal	1:20000	Sigma
Anti-HA Ab	Hemagglutinin	Rabbit polyclonal	1:1000	Santa Cruz
Anti-His Ab	Histidine	Mouse monoclonal	1:1000	Upstate
Anti-IFI27 Ab	IFI27	Mouse monoclonal	1:100-1:2000	Abcam
Anti-AICDA Ab	AICDA	Rat monoclonal	1:100:1:1000	Gift from Professor Niedobitek (Germany)

Table 2-4 Secondary antibodies were used for western blotting

Secondary	Conjugate	Dilution	Supplier
Goat anti-mouse IgG	Peroxidase	1:1000	Dako
Goat anti-rabbit IgG	Peroxidase	1:1000	Dako
Goat anti-rat IgG	Peroxidase	1:1000	Dako

2.5 Cell proliferation and viability assays

2.5.1 Cell proliferation assay

5 x 10^4 cells from each cell line were pelleted at 1500 rpm for 5 minutes and resuspended in 5 ml PBS. Cells were washed three times with PBS to remove any trace of serum. Finally, cells were resuspended in 2 ml of growth medium RPMI1640 containing glutamine, penicillin/streptomycin solution and 10%, 5%, or 1% B cell serum without G418 presence. 100 µl of cell suspension from each concentration of serum was pipetted in triplicate to 5 separate 96-well plates. The plates were labelled Day 0, 2, 4, 6 and 8 and incubated at 37° C in 5% CO_2 for up

to 8 days without changing the medium. Every 2 days (starting at Day 0), a plate was removed for WST-1 assay. Assessment of cell proliferation was carried out every two days (starting at Day 0) for serum concentration. Briefly 20 µl of WST-1 reagent (Roche) was added to each well and plates incubated for 1 hour at 37° C. Absorbances were read at 450 nm on a Microplate Autoreader (Model EL311; Bio-tek Instruments, Vermont, USA). Mean results were taken from triplicate wells and plotted as a graph showing standard error of mean, and subjected to statistical analysis in Microsoft™ Excel using a two-tailed student's T-test assuming the two samples displayed unequal variance. The p-values indicated a significant difference between the data sets when the p-value was <0.05.

2.5.2 Cell viability assay

1.2×10^6 cells from each cell line were pelleted at 1500 rpm for 5 minutes and resuspended in 5 ml PBS. Cells were washed three times to remove any trace of serum. Finally, cells were resuspended in 4 ml cell maintenance medium, RPMI1640 containing glutamine, penicillin/streptomycin solution and 10%, 5%, or 1% B cell serum without G418 presence. 200 µl of each cell suspension at each of the different serum concentrations was pipetted in triplicate into 5 separate 48-well plates. Plates were labelled Day 0, 2, 4, 6 and 8 and incubated at 37° C in 5% CO_2 for up to 8 days without changing the medium. Every 2 days (starting at Day 0), a trypan blue viability assay was carried out with trypan blue reagent (Sigma). 80 µl of cell suspension was mixed with 20 µl trypan blue reagent for 2 minutes and the cell viability was evaluated by direct counting of unstained cells under a microscope. Mean results were taken from triplicate wells and plotted as a graph showing standard error of mean, and subjected to statistical analysis in Microsoft™ Excel using a two-tailed student's T-test assuming the two samples

displayed unequal variance. The p-values indicated a significant difference between the data sets when the p-value was <0.05.

2.6 Gene expression microarray

2.6.1 RNA extraction

5-10x10^6 of KMH2-LMP2A, -LMP2B and -Neo cells were washed with PBS and pelleted. 1 ml TRIZOL Reagent was added to lyse the cells by repetitive pipetting. After 5 minutes incubation at room temperature (RT), 200µl chloroform was added and shaken vigorously for 15 seconds by hand. The mixture was incubated at RT for another 2-3 minutes then centrifuged at full speed for 15 minutes at 4°C. The aqueous phase was transferred into a fresh tube and 500µl of isopropyl alcohol was added to each sample. Samples were incubated for 10 minutes at RT, and then spun down at full speed at 4°C for 10 minutes. The supernatant was carefully removed and 1ml of 70% ethanol was added and mixed. The sample was centrifuged at 8000rpm for 5 minutes at 4°C and the supernatant was taken off. The RNA pellet was air dried for 10 minutes and dissolved in RNase-free water at 55°C for 10 minutes. RNA was stored at -80°C.

2.6.2 First strand cDNA synthesis

Reactions were made up in RNase free tubes and contained 10µg total RNA from KMH2-LMP2A, -LMP2B or-Neo, 2µl diluted poly-A control (Affymetrix), 1µl (100pmol/µl) T_7 $(dT)_{24}$ primer (Proligo, 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3'), plus DEPC treated dH_2O (Ambion) to a final volume of 11µl.The sample was mixed by pipetting and given a short spin, then incubated at 65-70°C for 10 minutes and put on ice. To each reaction was added 7µl of a master mix prepared

on ice with 4µl (5x) First Strand Buffer (Invitrogen), 2µl DTT (0.1M, Invitrogen), 1µl dNTPs (10mM, Invitrogen). After 2 minutes incubation, 1µl Superscript II Reverse Transcriptase (200U/ µl, Invitrogen) was added and incubated at 42°C for 1 hour. Samples were then ready to proceed to "Second Strand Synthesis" or stored at –80°C.

2.6.3 Second strand cDNA synthesis

A master mix was prepared on ice in a new RNase free microfuge tube with 91µl DEPC treated dH_2O, 30µl (5x) Second Strand Buffer, 3µl dNTPs (10mM, Invitrogen), 1µl E. *coli* DNA Ligase (10U/µl, Invitrogen), 4µl *E. coli* DNA Polymerase I (10U/µl, Invitrogen), 1µl *E. coli* RNAse H (2U/µl, Invitrogen) for each reaction. 130µl master mix was added to each "First Strand cDNA" reaction and incubated at 16°C for 2 hours. 2 µl T_4 DNA Polymerase (5U/µl, Invitrogen) was added to each reaction and incubated at 16°C for 5 minutes. Finally, 10µl 0.5M EDTA (Invitrogen) was added to each reaction after which the samples were ready to proceed to "Clean-up of double stranded cDNA" or were stored at –80°C.

2.6.4 Clean-up of double stranded cDNA

The Affymetrix Sample Cleanup Module was used to clean up cDNA following the instructions of the manufacturer. cDNA reaction mix was transferred to a 1.5ml tube and 600µl cDNA binding buffer was added. 500µl of the mixture was loaded to a cDNA cleanup spin column and spun for 1 min at full speed and the flow-through was discarded. The remainder of the mixture was loaded and spun as before. The column was transferred to a new collection tube and 750µl of wash buffer was applied to the column, which was spun at full speed and the flow-through was discarded. The column was centrifuged for 5min at maximum speed

with the cap opened. The column was transferred to a new 1.5ml collection tube. 14µl cDNA Elution buffer was directly loaded onto the spin column membrane and incubated for 1 min at room temperature, then spun for 1 min at full speed. Cleaned cDNA was ready for the *in vitro* transcription reaction or store at -80°C.

2.6.5 Synthesis of cRNA by *in vitro* transcription

An Affymetrix in vitro transcription (IVT) labelling kit was used for this step. All reagents and double stranded cDNA were thawed at room temperature. A master mix was prepared at room temperature containing, per reaction, 14µl DEPC treated dH_2O, 4µl 10x IVT labelling Buffer, 12µl IVT labelling NTP mix, 4µl IVT labelling enzyme mix. 34µl of the master mix was added into each 6µl of previously prepared cDNA from KMH2-LMP2A, -LMP2B or -Neo and incubated at 37°C overnight. Samples were then ready to proceed to "Clean-up of cRNA" or were stored at –80°C.

2.6.6 Clean-up of cRNA

The Affymetrix Sample Cleanup Module was used to clean up the cRNA following the instructions of the manufacturer. 60µl RNase-free water was added to the IVT reaction and mixed. 350µl cRNA binding buffer was added, mixed, and then topped up to 250µl 100% ethanol. The mixture was loaded to the IVT cRNA cleanup column and spun at full speed for 15 seconds. Flow-through was discarded and the spin column was transferred to a fresh 2ml collection tube. The column was washed with 500µl cRNA wash buffer and spun for 15 seconds at full speed. 500µl of 80% ethanol was applied to the column and spun for 15 seconds at full speed. The column was spun for 5minutes at maximum speed. 11µl of RNase-free water was loaded directly onto the column membrane and after I

minute incubation was spun down for 1 minute at full speed. cRNA was measured using the Nanodrop spectrophotometer. cRNA was ready for the fragmentation step or stored at -80 ° C.

2.6.7 cRNA fragmentation

A 50µl reaction mix was made up in a PCR tube with 10µl (5x) Fragmentation buffer (200mM Tris-acetate, pH 8.1, 500mM KOAc, 150mM MgOAc), 25µg cRNA and DEPC treated dH_2O. The samples were incubated in a PCR machine at 94°C for 35 minutes and put on ice immediately after the incubation. The samples were then ready to proceed to hybridization or stored at –80°C. An aliquot of the RNA, cRNA and fragmented cRNA of KMH2-LMP2A, -LMP2B or-Neo was run on a gel to check the RNA quality.

2.6.8 Hybridisation

Stocks of GeneChip Eukaryotic Hybridisation Controls, B2 oligo (provided with the GeneChip Eukaryotic Hybridisation Control kit, Affymetrix) were heated to 65 ^{0}C for 5 minutes before aliquoting and use. 40µl (20µg) of fragmented cRNA from KMH2-LMP2A, -LMP2B or -Neo was added to 360µl of hybridization cocktail containing 7µl of B2 Oligo, 20x Hybridisation Cocktail (Affymetrix), 4µl of Herring Sperm DNA (10mg/ml, Promega), 4µl of Acetylated BSA (50mg/ml, Invitrogen), 2x Hybridisation Buffer, 40µl DMSO (Sigma-Aldrich), and 85µl of DEPC treated dH_2O.

U133 plus2 chips (Affymetrix) were warmed to room temperature before use. They were then filled with 250 µl of 1x Hybridisation Buffer (see appendix 8.2.6) and incubated in the oven at 45°C for 10 with rotation at 60rpm.

The hybridisation cocktail was heated in a heating block at 99°C for 5 minutes then moved to a 45°C block for another 5 minutes. The samples were then spun at maximum speed in a microcentrifuge for 5 minutes. The buffer was removed from the pre-hybridised chips and replaced with 200µl of clarified hybridisation cocktail of KMH2-LMP2A, -LMP2B or-Neo. Arrays were incubated at 45°C for 16 hrs with rotation at 60rpm.

2.6.9 Washing and staining in the Fluidics station and scanning

The washing, staining and scanning procedures were controlled using Affymetrix Gene Chip Operating Software (GCOS). The washing and staining process used an antibody amplification protocol and comprised 3 staining steps: 1st: SAPE (Streptavidin Phycoerythrin), 2nd: Antibody (biotinylated α-streptavidin) and 3rd: SAPE. SAPE was the fluorescent stain used and was stored in the dark at 4°C (in an amber tube). The SAPE stain solution was prepared fresh immediately before use.

2.6.9.1 Preparation of the SAPE stain and antibody stain solutions

1st and 3rd stain solution were the same. 600µl of SAPE stain was prepared for each array containing 300µl of 2x MES Stain buffer (Sigma, see Appendix 8.2.6), 24µl of Acetylated BSA (50mg/ml, Invitrogen), 6µl of SAPE (1mg/ml, Cambridge biosciences) and 270µl of DEPC dH_2O.

600 µl of antibody solution for each array contained 300µl of 2x MES stain buffer, 24µl of Acetylated BSA (50mg/ml), 6µl of Normal goat IgG (10mg/ml, sigma), 3.6µl of α-streptavidin biotinylated antibody (goat, 0.5mg/ml, Vector Labs) and 266.4µl of DEPC H_2O.

2.6.9.2 Washing and staining

After16 hrs incubation, the hybridisation cocktail was removed from the chips and stored at -80°C for long-term storage. The array was filled with 250µl of Non-Stringent Wash Buffer A (See Appendix 8.2.6) (The array can be stored at 4°C for 3 hrs at this step if necessary). Arrays were washed and stained on an Affymetrix FS400 fluidics station using protocol EukGE_WS2v5.

2.6.9.3 Scanning

Arrays were scanned using an Affymetrix Gene Chip Scanner 3000. GCOS software generates the primary scan intensity data (.dat) files which are then automatically converted into "smoothed" .cel files that can be further analysed by GCOS or other array analysis packages.

2.6.10 Statistical significance of differential expression

The Affymetrix system of gene expression quantification was used. Three biological replicate experiments comparing KMH2-Neo gene transcription with either KMH2-2A or KMH2-2B cells were performed. Following hybridisation and scanning of arrays, scanned image of microarray chips were analysed using the GCOS (GeneChip Operating Software) from Affymetrix, Inc. (Santa Clara, California, USA) with the target signal set to 100. Probe level quantile normalisation (Bolstad *et al.*, 2003) and RMA (robust multi-array analysis) (Irizarry *et al.*, 2003) were preformed using the affymetrix package of the Bioconductor project (http://www.bioconductor.org). Differentially expressed probe sets were identified using the rank products method (Breitling *et al.*, 2004; Breitling and Herzyk 2005) of the RankProd package in Bioconductor following RMA with the PFP (percentage of false-positives) threshold set to 10%. Gene

expression heat maps were generated using dChip (http://www.dchip.org) with the default settings. Genes with changes in differential expression values of two fold or greater, probability values less than 0.05, and Affymetrix calls other than no change were considered statistically significant.

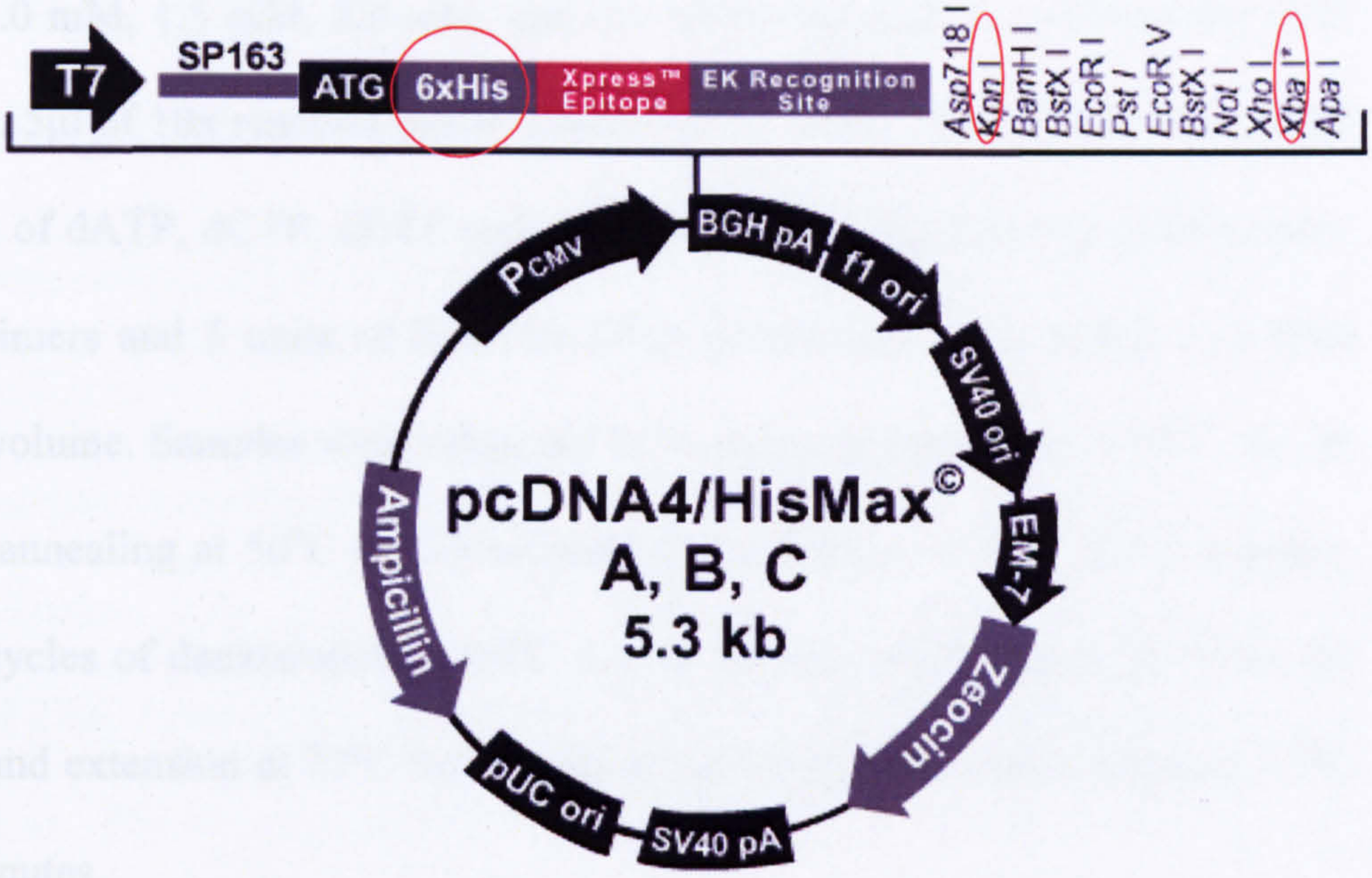
2.7 Cloning of DNA in bacterial plasmids

2.7.1 Design primers using software Prophet

Expression vector pCDNA4His/maxA (Invitrogen), containing a polyhistidine tag, CMV promoter, Zeocin™ resistance gene, Enterokinase recognition site, Multiple cloning sites, Ampicillin resistance gene, SV40 promoter and origin, was used for making LMP2 plasmids. LMP2A, LMP2A-N and LMP2B primers were designed using software Prophet including the two cloning site sequences of the vector. Two restriction sites, *KpnI* and *XbaI* were selected and the sequence of *KpnI* (GGTACC) was added to forward primers and *XbaI* (TCTAGA) to reverse primers. The map of the vector used for making the plasmids is given in Figure 2.1.

Figure 2-1 Map pcDNA4/HisMax A vector

The expression vector, pcDNA4/HisMax A, was used to make all three LMP2 constructs, LMP2A, LMP2B and LMP2A-N. 6-polyhistidine tag is circled in red as are restriction sites for *Kpn* I and *Xba* I in the map of the vector.



Comments for pcDNA4/HisMax© A:
5258 nucleotides
CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
QBI SP163 translational enhancer: bases 917-1079
ATG initiation codon: bases 1080-1082
Polyhistidine tag: bases 1092-1109
Xpress™ epitope: bases 1149-1172
Enterokinase recognition site: bases 1158-1172
Multiple cloning site: bases 1172-1245
BGH reverse priming site: bases 1265-1282
BGH polyadenylation sequence: bases 1268-1495
f1 origin: bases 1541-1969
SV40 promoter and origin: bases 1996-2305
EM-7 promoter: bases 2353-2408
Zeocin™ resistance gene: bases 2427-2801
SV40 polyadenylation sequence: bases 2931-3061
pUC origin: bases 3444-4117
Ampicillin resistance gene: bases 4262-5122

2.7.2 PCR for LMP2A, LMP2B and LMP2A-N

1ng of pSG5-LMP2A plasmid DNA (kindly provided by Professor Richard Longnecker, North Western University, Chicago) was used as a template for PCR amplification of LMP2A, LMP2B and LMP2A-N. Different concentrations of $MgCl_2$, 1.0 mM, 1.5 mM, 2.0 mM, and 2.5 mM were used to optimise the PCR condition. 5µl of 10x reaction buffer (50mM KCl, 10mM Tris-HCl, pH 8.3), 0.25 mM each of dATP, dCTP, dGTP and dTTP, 1.0 µM each of forward and reverse LMP2 primers and 5 units of Red Hot DNA polymerase were added in a 50µl reaction volume. Samples were subjected to 5 cycles of denaturing at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 2 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 2 minutes with a final extension phase at 72°C for 10 minutes.

2.7.3 Purification of PCR products

100µl of PCR products were purified using PCR purification Kit (Qiagen); following the protocol provided by the manufacturer. DNA was eluted in 50µl elution buffer, and then measured using a UV-spectrophotometer.

2.7.4 Restriction of vector and LMP2 DNA

3 x 3µg of pCDNA4His/maxA vector DNA and 3µg of each of LMP2A, LMP2B or LMP2A-N DNA were aliquoted to labelled 0.5ml tubes. The restriction reaction contained 4µl of 10x restriction buffer, 20 units of each enzyme, *KpnI* and *Xbal* and dH_2O to 40µl. The samples were incubated at RT overnight. Results were checked by running 1.5% agarose gels and visualised under UV light. Correctly size bands of DNA were cut for purification.

2.7.5 Purification of restricted DNA

Sterile empty eppendorfs were weighed and labelled. Bands were cut under UV light and placeed into the corresponding tubes. DNA was purified using a gel purification kit (Qiagen) according to the protocol provided by the manufacturer. DNA was eluted in 25µl elution buffer, measured using a spectrophotometer and prepared for dephosphorylation and ligation.

2.7.6 Dephosphorylation of vector DNA using Shrimp Alkaline Phosphatase (SAP)

15µl of purified vector DNA was added to a 0.5 ml eppendorf tube on ice with 2.7 µl SAP (Roche), 2.7µl of 10 x buffer and 6µl of sterile dH_2O to make a total volume of 27µl. After mixing and a short centrifugation, the reaction mixture was incubated at 37°C for 1 hour, followed by 65°C for 15minutes to denature the enzyme. The vector was ready for ligation.

2.7.7 Ligation

50ng of dephosphorylated vector was used for each reaction. The volume of purified DNA from LMP2A, LMP2B and LMP2A-N calculated according to their molecular weight and the equation below were placed into the corresponding 0.5ml tubes on ice. 10µl of 2x buffer plus water were added to each 20µl reaction. The reactions were incubated at RT for 15 minutes, and then at 4°C overnight.

Equation 2-1 inserted DNA is required:

(ng) vector x (Kb) insert / (Kb) vector x 3

2.7.8 Transformation

DH5α bacteria were thawed and the ligated DNA chilled on ice. PUC19 plasmid DNA (Invitrogen, working concentration 50ng/μl) was used as a positive control. 50μl of bacteria were added to each tube together with 20μl of ligated DNA. Samples were mixed and chilled on ice, then incubated at 42°C water bath for 90 seconds and put back on ice immediately. 500μl L-broth solution (10g L-Broth powder in 500 ml distilled H_2O, autoclaved) was added to each tube; mixed and incubated at 37°C for 1 hour (vortexed 2-3 times). LB plates (10g L-Broth powder and 7.5g of Nutrient Agar in 500 ml distilled H_2O, autoclaved) with Ampicillin (100μg/ml) were put into a 37°C incubator (the lids were left slightly open to allow dissipation of steam). 200μl of each reaction solution was plated onto its corresponding plate under flame with a sterile glass rod. All the plates were incubated upside down in a 37°C incubator overnight.

2.7.9 Cloning

Clones were checked and selected the next day. 3ml of LB solution containing Ampicillin was added to 15ml tubes and labelled. Big, round single clones were picked under flame with sterile pipette tips and put into the tubes. All tubes were put into a 37°C shaker and left overnight.

2.8 Amplification and purification of plasmid DNA

2.8.1 Small scale preparation of plasmid DNA

1.5ml of each of the bacteria culture solution was poured into an eppendorf and centrifuged at full speed for 2 minutes at 4°C. Supernatant was removed by aspiration and the bacterial pellet was left as dry as possible. The bacterial pellet was resuspended in 100μl of ice-cold solution I (See Appendix 8.2.1) by vigorous

vortexing. 200µl of freshly prepared solution II (See Appendix 8.2.1) was added and the tube was closed tightly, mixed by inverting the tube rapidly five times and stored on ice. 150µl of ice-cold solution III (See Appendix 8.2.1) was added and vortexed gently in an inverted position for 10 seconds to disperse Solution III through the viscous bacterial lysate. The tube was stored on ice for 10 minutes. Samples were centrifuged at full speed for 5 minutes at 4°C, and then the supernatant was transferred into a fresh tube. 210µl of phenol and 210µl of chloroform was added and mixed by vortexing. After centrifuging at full speed for 2 min at 4°C in a microcentrifuge, supernatant was transferred into a fresh tube. The double-stranded DNA was precipitated with two volumes of absolute ethanol (about 900µl), mixed by vortexing and put into -20°C freezer for 30 minutes. Samples were centrifuged at full speed for 5 minutes in a 4°C microcentrifuge and the supernatant was removed by gentle aspiration. The pellet of double-stranded DNA was rinsed with 1ml of chilled 75% ethanol. The supernatant was removed by gentle aspiration and the pellet of DNA was left to dry in the air for 10 minutes. The DNA was redissolved in 50µl of elution buffer (TE buffer). DNA was ready for sequencing.

2.8.2 Large scale preparation of plasmid DNA

For this the Plasmid Maxi Prep 500 Kit (Qiagen) was used. A single colony of transformed bacteria was picked and grown overnight in a 2 ml shaking culture of L-Broth containing Ampicillin (50µg/ml) and incubated overnight at 37°C. 250µl of this initial starter culture was then used to inoculate 250mls of L-broth with ampicillin (50µg/ml) and this culture incubated overnight at 37°C. The bacterial culture was centrifuged at 6000rpm for 15 minutes at 4°C. The supernatant was decanted off and the bacterial pellet resuspended in 10 ml of buffer P1. The

suspension was then incubated with 10ml of buffer P2 and mixed by inverting 4-6 times and incubated at room temperature for 5 minutes. To this suspension 10 ml of chilled buffer P3 was added and gently mixed by inverting 4-6 times and incubated on ice for 20 minutes. The suspension was centrifuged at 12,000rpm for 30 minutes at 4°C. The supernatant was removed and centrifuged at 12,000 rpm for another 15 minutes at 4°C. The supernatant was then harvested. A Qiagen-tip 500 was equilibrated with 10ml of buffer QBT and allowed to flow through by gravity flow. The flow through was discarded and the column was washed twice with 30 ml of buffer QC. The flow through was discarded and the DNA eluted from the column with 15ml of buffer QF. The DNA was precipitated from the supernatant by adding 10.5ml of room temperature isopropanol. This was mixed and centrifuged at 11,000rpm for 30 minutes at 4°C. The supernatant was then carefully removed without disturbing the pellet. The DNA pellet was washed with 5 ml of 70% ethanol and centrifuged at 11,000rpm for 10 minutes. The supernatant was again carefully removed without disturbing the pellet. The pellet was then allowed to air dry for 5-10 minutes and redissolved in TE buffer. The DNA concentration and quality was then checked using a spectrophotometer (Genequant, Pharmicia)

2.9 DNA Sequencing

2.9.1 Labelling with fluorescent dye and precipitation of products

Sequencing was performed using the 3100 capillary sequencer from PE Applied Biosystems. The labelling reactions were set up using 30-50ng of PCR products, 1ul (3.2pmol) primer, 2µl of Big-Dye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied biosystems) and 6µl of 2.5x sequencing buffer (200

mM Tris HCl,5mM $MgCl_2$ pH 9.0). The labelling PCR was carried out for 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The labelled products were precipitated with 50µl of 95%(v/v) ethanol and 2µl of 3M sodium acetate pH 4.6 in 0.5 ml tubes at room temperature for 30-60 minutes. The samples were then micro-centrifuged at full speed for 20 minutes at 4°C. The supernatant was removed and the pellets washed by adding 250µl 70% (v/v) ethanol and gentle flicking followed by spinning at full speed for 5 minutes. The supernatant was then removed and the pellets dried for 30-60 minutes. 10µl of Hi-Dye™ (PE Applied Biosystems) was added and the pellet dissolved.

2.9.2 The 3100 ABI prism™ DNA capillary sequencer (PE Applied Biosystems) Samples were denatured at 95°C for 5 minutes and put on ice for 2 minutes before being left at RT ready for use. The buffer chambers were first filled with 2.5x EDTA buffer (PE Applied Biosystems) and the water chambers filled accordingly. 3100 POP 6™ Performance Optimized Polymer was used to fill the 50cm sequencer capillary array (PE Applied Biosystems). The 3100 data collection software version 1.0 was set for sequencing at: Dye Set, Z; Mobility file, DT3100POP6 (BD) v3.mob; BIOLIMS: Project, 3100_Project1; RunModule1, StdSeq50_POP6DefaultModule and analysis Module 1, BC-3100_SeqOff.saz. The samples were loaded into the appropriate 96 well plates and run for approximately 2.5 hours. Following the run, the extracted samples were analysed using the ABI sequencing analysis version3.6.1 software and results were checked using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/)

2.10 Transfection of mammalian cells with plasmid DNA

2.10.1 Transfection of LMP2 plasmids into 293 cells using lipofectamine 2000 and Zeocin drug selection

293 cells were maintained in RPMI DMEM-Hepes medium supplemented with 10% FCS, 2mM L-glutamine, and 1% penicillin/streptomycin solution. The day before transfection, 293 cells were trypsinized, counted and plated into 6-well plates to make sure that the confluence of the cells would be 90% on the day of the transfection. Antibiotics were avoided during the cell plating. The DNA and lipofectamine reagent were diluted with Opti-MEM^{R} I Reduced-Serum Medium (Invitrogen) in separate tubes and incubated at RT for 15 minutes. The diluted DNA and lipofectamine reagent were combined and mixed well, then incubated at RT for 20 minutes. Mixed complexes were gently added into the corresponding cell medium and incubated at 37°C for 3 hours. After 3 hours incubation, the fresh medium was topped up to bring the final concentration of serum to normal growth medium. The day after transfection, cells were passaged into fresh medium and the following day, Zeocin (500μg/ml, Invitrogen) was added to select the stably transfected cells. It took several weeks to select the 293 cells stably expressing LMP2.

2.11 Purification of LMP2 proteins with Ni-NTA beads

LMP2A-transfected 293 cells were washed twice in ice-cold PBS and then collected by scraping into 3 ml ice-cold PBS. The cells were pelleted by centrifugation at 1500rpm for 5 minutes at 4°C. The cell pellet was lysed in 1% Triton lysis buffer (pH 8.0) in ice for 30 minutes, and the cell debris pelleted by centrifugation at 13000rpm for 10 minutes at 4°C. Protein concentration was

determined using the Biorad protein assay. Ni-NTA beads were washed twice with pH 8.0 washing buffer with 20mM imidazole. 1mg protein was incubated with 100 µl of 50% v/v washed Ni-NTA beads at 4°C with rotation for 2 hours. The samples were then centrifuged briefly to pellet the Ni-NTA beads and the flow-through supernatants transferred to fresh tubes for immunoblotting. The beads were washed three times with the same washing buffer. Following the final wash, the beads were drained with a fine gauge needle and then the proteins were eluted with 100µl of pH 8.0 elution buffer with different concentrations of imidazole (100mM, 150mM, 200mM, 250mM) and 0.1% EDTA (pH 8.0). 20µl of each eluted protein was loaded for immunoblotting and the membrane was blotted with anti-His antibody (1:1000). To reduce elution of non-specific proteins, the concentration of imidazole in washing buffer was increased to 50mM.

2.12 Stable isotope labelling with amino acids in cell culture (SILAC)

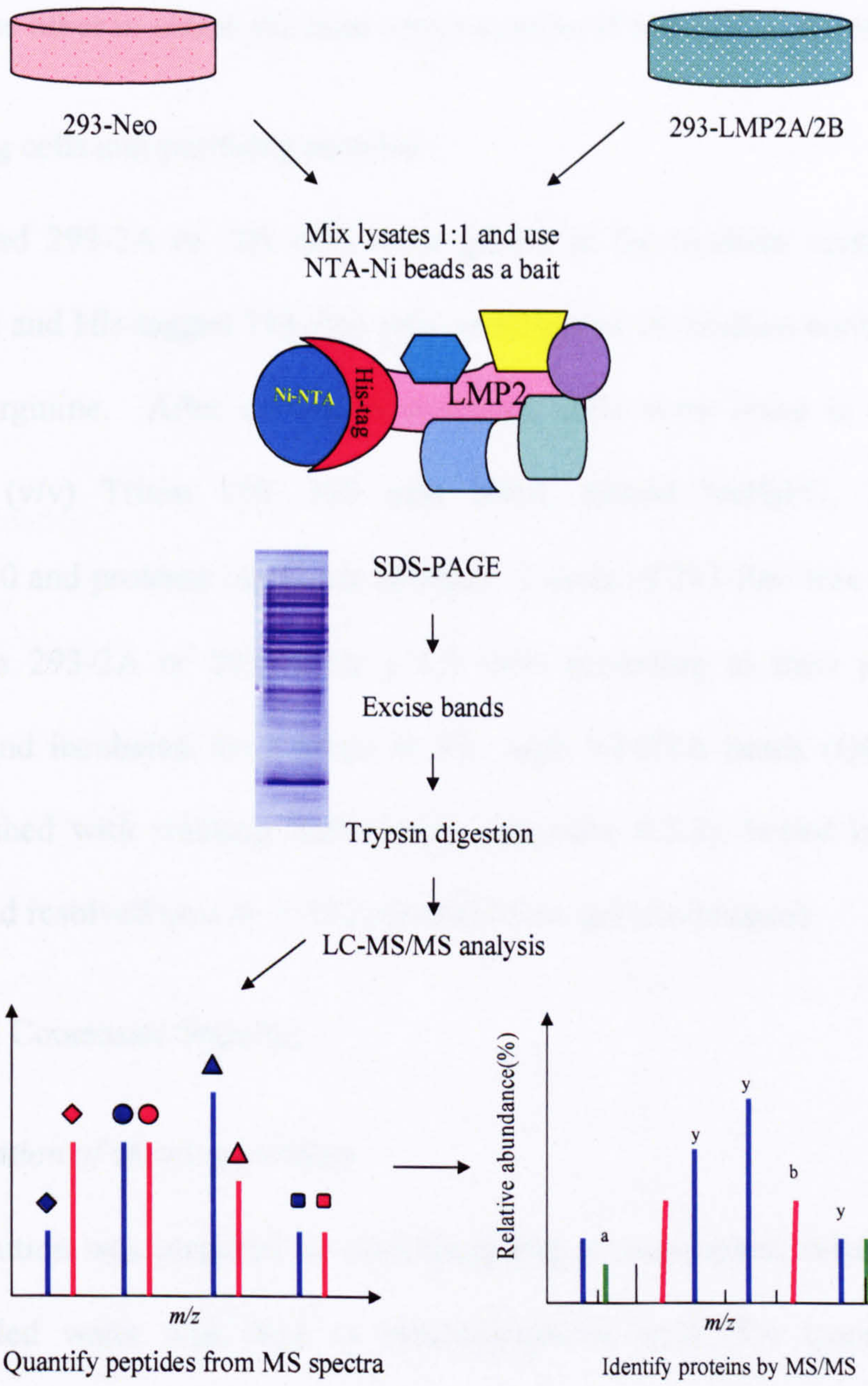
2.12.1 The principle of SILAC

293-Zeo cells and 293-2A or 293-2B cells were cultured in media containing [$^{12}C_6$]-Arginine and [$^{13}C_6$]-Arginine, respectively and protein lysates were made. His-tagged protein from 293-Zeo were mixed at a 1:1 ratio with protein from 293-2A or 293-2B, creating two samples that were then purified with Ni-NTA beads. Ni-NTA was used as a bait to enrich His-tagged LMP2 proteins and its binding partners. Eluted proteins were boiled in SDS-PAGE sample buffer, resolved on a 4-21% Tris-glycine gel, and lightly stained using Coomassie Blue. The gel lane was excised and cut horizontally into 10 sections of similar size. Proteins were digested by trypsin; peptides were extracted with 5% formic acid and analysed by mass spectrometry. The ratio of peptide intensities produced from the light and

heavy peptides in the MS spectra were determined. The fragmentation pattern of the ions in the MS/MS analysis and the mass of the parent peptide were then used to search human and EBV databases from NCBI using TurboSequest search engines to identify the peptide sequence and to assign the peptide to a particular protein. The 6 Da increase in the mass of the heavy peptides was taken into account in the search by including a 6 Da modification on the Arginine residues. Protein abundances were calculated as ratios of the areas of the monoisotopic peaks of the light *versus* the heavy peptides. An outline of the SILAC method is shown in Figure 2.2

Figure 2-2 Schematic of the SILAC method

293-Zeo cells and 293-2A or 293-2B cells were cultured in media containing [12C6]-Arginine and [13C6]-Arginine, respectively and protein lysates were made. His-tagged protein from 293-Zeo were mixed at a 1:1 ratio with protein from 293-2A or 293-2B, creating two samples that were then purified with Ni-NTA beads. Ni-NTA was used as a bait to enrich His-tagged LMP2 proteins and its binding partners. Eluted proteins were resolved on SDS-PAGE gel and lightly stained using Coomassie Blue. 10 excised sections of gel were digested by trypsin; peptides were extracted with 5% formic acid and analysed by mass spectrometry. The fragmentation pattern of the ions in the MS/MS analysis and the mass of the parent peptide were then used to search human and EBV databases from NCBI using TurboSequest search engines to identify the peptide sequence and to assign the peptide to a particular protein. The 6 Da increase in the mass of the heavy peptides was taken into account in the search by including a 6 Da modification on the Arginine residues.


(12C)ARG
(13C)ARG
293-Neo
293-LMP2A/2B
Mix lysates 1:1 and use
NTA-Ni beads as a bait
Ni-NTA
His-tag
LMP2
SDS-PAGE
Excise bands
Trypsin digestion
LC-MS/MS analysis
Relative abundance(%)
m/z
Relative abundance(%)
y
y
b
y
a
m/z
Quantify peptides from MS spectra
Identify proteins by MS/MS

2.12.2 Preparing medium

5ml glutamine (Sigma) and 75ml dialysed FCS (Invitrogen) was added to 500ml of DEME+HEPES medium (no Arginine, Sigma) and mixed. The medium was split into two labelled sterile bottles. 29mg of normal [$^{12}C_6$]-Arginine (Cambridge Isotope Inc) was added to one bottle and 29mg of [$^{13}C_6$]-Arginine (Cambridge Isotope Inc) to the other to obtain the final concentration of 100 mg/L arginine.

2.12.3 Labelling cells and purifying proteins

2 x10^8 His-tagged 293-2A or -2B cells were grown in the medium containing [$^{13}C_6$] - Arginine and His-tagged 293-Zeo cells were grown in medium containing normal [$^{12}C_6$]-Arginine. After at least 5 passages, cells were lysed in buffer containing 1% (v/v) Triton 100, 300 mM NaCl, 50mM NaH_2PO, 10mM imidazole, pH 8.0 and protease inhibitors (Roche). Lysate of 293-Zeo was mixed with lysate from 293-2A or 293-2B in a 1:1 ratio according to their protein concentrations and incubated for 2 hours at 4°C with Ni-NTA beads (Qiagen). Beads were washed with washing buffer (See Appendix 8.2.5), boiled in SDS sample buffer and resolved on a 4–21% (wt/vol) Novex gel (Invitrogen).

2.12.4 Colloidal Coomassie Staining

2.12.4.1 Preparation of staining solution

The staining solution was prepared by dissolving 40g of ammonium sulphate in 384ml of distilled water with 8ml of orthophosphoric acid. 5% Coomassie Brilliant Blue G250 (Sigma) was prepared by mixing thoroughly 0.5g of dye and 10ml of distilled water. 8ml of this dye solution was added to the ammonium sulphate/phosphoric acid. The final concentration was 0.08% Coomassie Brilliant

Blue G250, 1.6% Orthophosphoric Acid, 8% Ammonium Sulphate and 20% Methanol.

2.12.4.2 Coomassie Staining and destaining

After the gels were run they were placed directly into freshly prepared staining solution, which also fixes the proteins. The gel was placed on a shaking platform in a closed box at RT and left to stain overnight. After the staining solution was poured off, the gel was rinsed twice with water to remove excess stain and then placed in 1% acetic acid, which was changed regularly to speed up destaining. The gel background became completely clear eventually, whilst the proteins remained blue.

2.12.5 In gel digestion

2.12.5.1 Water wash

The bands were excised and placed into the pre-washed Eppendorf tubes (rinsed with 0.1% formic acid dissolved in 50:50 water:acetonitrile and dried down), and 300µl of distilled/deionised water added. After 15 min the water was removed carefully using a pipette with a fine tip. This step removes the acetic acid contained in the destaining solution.

2.12.5.2 Wash and pH equilibration

100µl of 50mM ammonium bicarbonate in 50% acetonitrile was added to each tube and incubated, with shaking for 45 min at 37°C. The above step was repeated twice to ensure complete equilibration. After the supernatant was removed the gel was dried down using a vacuum rotary evaporator to remove the liquid from the gel.

2.12.5.3 Reduction Step, carboxymethylation and Wash

50µl of 50mM DTT (made up fresh in 100mM ammonium bicarbonate in 10% acetonitrile) was added to the dried gel and incubated for 1 hour at 60°C and the supernatant removed. 50µl of 100mM iodoacetamide (made up fresh in 100mM ammonium bicarbonate in 10% acetonitrile) was added and the tube was incubated for 30 minutes at RT in the dark. The supernatant was removed and the gels washed as follows; 100µl of 40mM ammoniun bicarbonate in 10% acetonitrile was added and incubated for 15 minutes at RT, with shaking and then the supernatant removed. This wash was performed 3 times before the gel was completely dried again using a vacuum rotary evaporator.

2.12.5.4 Trypsinization

The gel was rehydrated using 40µl of 12.5µg/ml sequencing grade modified trypsin (Promega) dissolved in 40mM ammonium bicarbonate/10% acetonitrile for 1 hour at RT. After the gel was completely re-swelled, 40µl of 40mM ammonium bicarbonate in 10% acetonitrile was added and incubated at 37°C overnight.

2.12.5.5 Harvesting the Tryptic Peptides

The supernatant was carefully removed and replaced with 30µl of 3% formic acid in water, incubated for an hour at 37°C and this solution collected and added to the initial supernatant. Another 30µl of 3% formic acid in water was added and after incubation for 1 hour, the final supernatant was harvested and pooled with the other two. The approximate volume of the combined supernatants was 100µl. This solution was ready for direct analysis using an LC-MS/MS based system.

2.12.6 Mass Spectrometric analysis

85µl of the peptides were separated on a 75µm i.d. 15cm long C18 PepMap reverse phase column (3 µm particle size) (Dionex) using a 5% - 37.5% solvent B gradient over 40min (solvent A=5% acetonitrile/0.1% formic acid and solvent B= 95% acetonitrile/0.1% formic acid) at 200nL/min using a Dionex/LC Packings Famos/Switchos/Ultimate nanobore HPLC system. The separated peptides were sprayed, using electrospray ionization, directly into a ThermoFinnigan LCQ Deca XP Plus ion-trap mass spectrometer via a nasnospray source equipped with a 10 µM uncoated emmiter tip. A charge of 1.3Kv was applied directly to the liquid stream and the mass of the peptide determined in the initial MS analysis and this data was used to select ions for MS/MS analysis. The most abundant 5 ions for each full scan were analysed sequentially before another full scan was performed. The fragmentation spectra were analysed using TurboSequest as part of the BioWorks 3.1 suite of data analysis programs and Xcalibur software package.

CHAPTER THREE: GENERATION AND VALIDATION OF LMP2A OR LMP2B EXPRESSING HODGKIN'S LYMPHOMA CELL LINE

3 Generation and validation of LMP2A or LMP2B expressing Hodgkin's lymphoma cell lines

3.1 Introduction

Hodgkin's lymphoma differs from other lymphomas because it is characterized by rare tumour cells that are surrounded by many more infiltrating cells of the immune system (Kanzler *et al.*, 1996; Weiss et *al.*, 1999; Küppers and Hansmann, 2005). Approximately half of Hodgkin's lymphomas have EBV DNA within the tumour cells and LMP2 is consistently expressed in all EBV positive cases (Küppers *et al.*, 2004; Young and Rickinson, 2004). This suggests that LMP2 plays an important role in the pathogenesis of HL. However, the exact function of LMP2 in HL still remains unknown. Most research on LMP2 has been performed on other B cells and little has been undertaken on HL cells.

LMP2A is a transmembrane protein that functions to inhibit normal B-cell signal transduction by mimicking an activated B-cell receptor (BCR) (Miller *et al.*, 1995, Portis *et al.*, 2002). Paradoxically, LMP2A can provide survival signals to B cells *in vivo*, where expression of an LMP2A transgene in mice allows BCR-negative cells to exit the bone marrow and survive in peripheral lymphoid organs (Caldwell *et al.*, 1998). A recent DNA microarray study demonstrated that LMP2A induced alterations in cellular gene expression similar to those previously described to be characteristic of HRS cells. These included the downregulation of B cell lineage markers and transcription factors important for normal B-cell development, e.g. Syk, BLNK (Portis *et al.*, 2003; Schwering *et al.*, 2003). This suggests that LMP2A might make an important contribution to the pathogenesis of HL.

The study of the function of LMP2 in HL has been hampered by the lack of *in vitro* systems. Therefore in order to investigate the function of LMP2A and LMP2B in HL, it is important to generate the appropriate cell line models.

Most B lymphoma cell lines including those of HL cannot be transfected easily; this is especially true in the case of LMP2A and LMP2B, which are transmembrane proteins and toxic to cells. Several nonviral approaches have been attempted to enhance the transfection efficiency of lymphoma cells; include these the gene gun (Burkholder *et al.*, 1991), electroporation (Lenz *et al.*, 2003; Brielmeier *et al.*, 1998), Lipofection (Zelphati *et al.*, 2001,) and a novel electroporation-based technique, termed nucleofection (Thiel *et al.*, 2001). However, transfection efficiency is problematic for all these physical approaches (Brielmeier *et al.*, 1998, Coonrod *et al.*, 1997). It was reported that B-lymphoma cell lines and primary lymphoma cells can be efficiently transduced using an adenoviral vector system (Buttgereit *et al.*, 2000); this suggests this approach might be a feasible approach to obtain LMP2 expression in HL cell lines.

Retroviral packaging cell lines producing LMP2A, LMP2B, or the neomycin control retrovirus were used for the transduction of established epithelial cell lines (Allen *et al*, 2005; Dawson *et al.*, 2001). The advantages of using retroviral transduction over standard DNA transfection are several folds. First, the retroviral transduction is more efficient and more reliable than lipid or electroporation-based transfection methods. Second, the retroviral method produces a polyclonal population of cells that overcomes concerns over clonal selection. Third, and once the stable retroviral packaging cell lines are established, an unlimited supply of retrovirus can be obtained.

Aims of this study:

1. To generate HL cell lines stably expressing LMP2A and LMP2B.
2. To investigate the impact of LMP2A and LMP2B on cellular phenotype using growth and viability assays.

3.2 Results

3.2.1 Generation of LMP2A- and LMP2B-expressing KMH2 and L428 cell lines and confirmation of expression at the RNA level

The A13 stably transfected packaging cells with the retrovirus carrying vector pLNSX (Neo), pLNSX-LMP2A and pLNSX-LMP2B plasmids were seeded into 6cm Petri dishes and the recombinant retroviruses produced by these cells were shed into the surrounding media. The conditioned media containing retrovirus carrying empty vector pLNSX control (Neo), pLNSX-LMP2A or pLNSX-LMP2B were collected after 48 hours incubation, and used to transduce KMH2 and L428 cell lines. The cells stably transduced with retroviruses carrying Neo, LMP2A and LMP2B were grown in RPMI 1640 medium under pressure of G418 (1mg/ml) drug selection, along with parental KMH2 and L428 cells. Parental cells died off in one week of drug selection. 1 clone of Neo, 5 clones of LMP2A and 1 clone of LMP2B were generated from each HL cell line.

Table 3-1 Key to the nomenclature of cell lines generated in this study

Cell lines	pLNSX(Vector) (1 clone)	pLNSX-LMP2A (5 clones)					pLNSX-LMP2B (1 clone)
KMH2	K-Neo	K-2A1	K-2A2	K-2A3	K-2A4	K-2A5	K-2B
L428	L-Neo	L-2A1	L-2A2	L-2A3	L-2A4	L-2A5	L-2B

3.2.1.1 Confirmation of the expression of LMP2A in the transduced HL cell lines, KMH2 and L428 by RT-PCR

Transduced cells were harvested two weeks post infection. RNA was extracted and cDNA was made with a specific LMP2A reverse primer. The LMP2-positive LCL cell line, X50-7, was used as positive control. All 5 clones of infected KMH2 and L428 were LMP2A positive, but the expression level of LMP2A was variable; clones K-2A1, K-2A4 and K-2A5 showed higher levels of LMP2A expression than the others (Figure 3.1A). For L428 cells, clones L-2A1, L-2A4 and L-2A5 showed the strongest expression and clone L-2A2 the weakest one (Figure 3.1B and C). As expected, parental KMH2 and L428 cells showed no expression of LMP2A. X50-7 cells expressed LMP2A at higher levels when compared to all of newly infected KMH2 and L428 cells. RT-PCR for GAPDH showed equal loading for all samples.

RT-PCR for LMP2A was repeated 6 weeks post infection. Interestingly, this time the expression of LMP2A in all 5 clones of KMH2-LMP2A and L428-LMP2A was the same and stronger than at two weeks post infection. Furthermore, after 5 months the expression of LMP2A remained at the same level as that detected at 6 weeks. Thus, except after the initial phase when expression increased, the expression of LMP2A in the infected cell lines remains stable over time.

Figure 3-1 Expression of LMP2A in transduced HL cell lines, KMH2 and L428 two weeks post infection

RNA was extracted from 5 clones of LMP2A transduced KMH2 and L428 cells (K-2A1-5, L-2A1-5), A single clone of each empty vector transduced KMH2, L428 cells (K-Neo, L-Neo), parental KMH2, L428 cells, and X50-7 cells.

(A) All 5 clones of infected KMH2 and L428 cells were LMP2A positive, but the expression level of LMP2A was variable; clones K-2A1, K-2A4 and K-2A5 showed higher levels of LMP2A expression than the others.

(B and C) For L428 cells, clones L-2A1, L-2A4 and L-2A5 showed the strongest expression and clone L-2A2 the weakest. Parental KMH2, L428, K-Neo, L-Neo cells and negative controls showed no expression of LMP2A. X-50-7 cells expressed LMP2A at higher levels compared to all of newly infected KMH2 and L428 cells. RT-PCR for GAPDH was run at the same time as a loading control.


Marker
K-2A1
K-2A2
K-2A3
K-2A4
K-2A5
Negative
KMH2
X50-7
LMP2A
GAPDH
A


Marker
Negative
L-2A1
L-2A2
L-2A3
X50-7
LMP2A
GAPDH
B

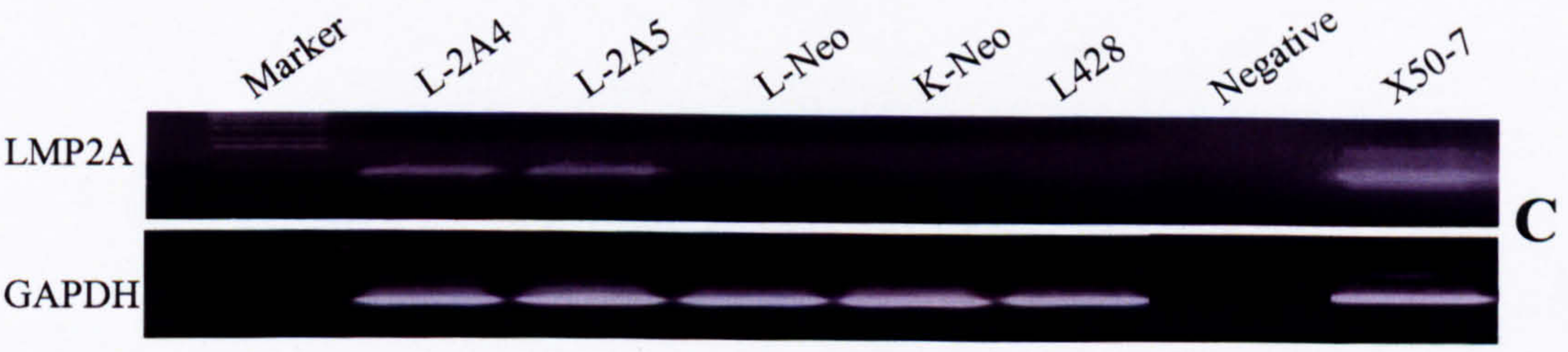

Marker
L-2A4
L-2A5
L-Neo
K-Neo
L428
Negative
X50-7
LMP2A
GAPDH
C

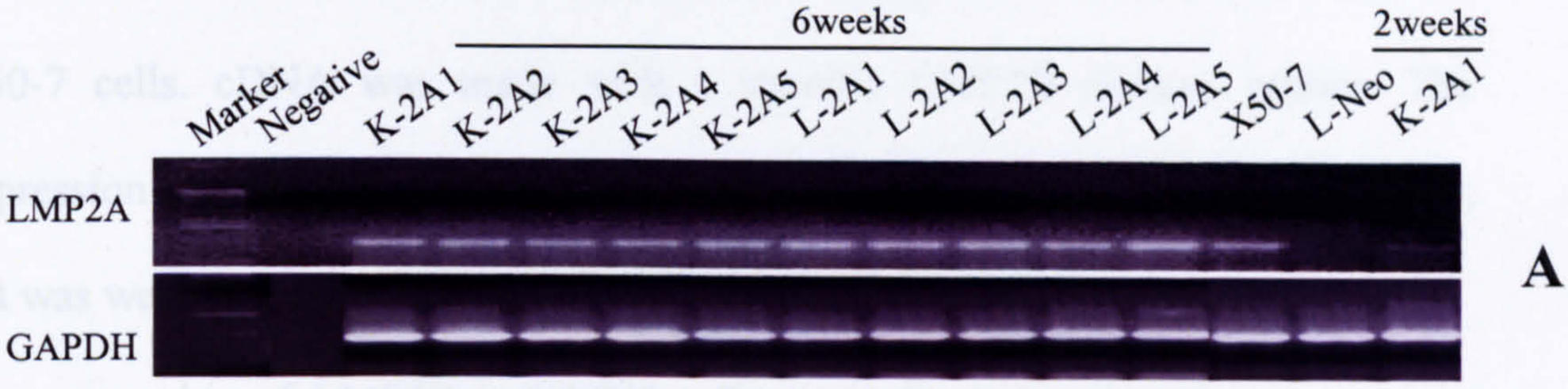
Figure 3-2 Comparison of LMP2A expressing KMH2 and L428 cells at 6 weeks and 5 months post infection

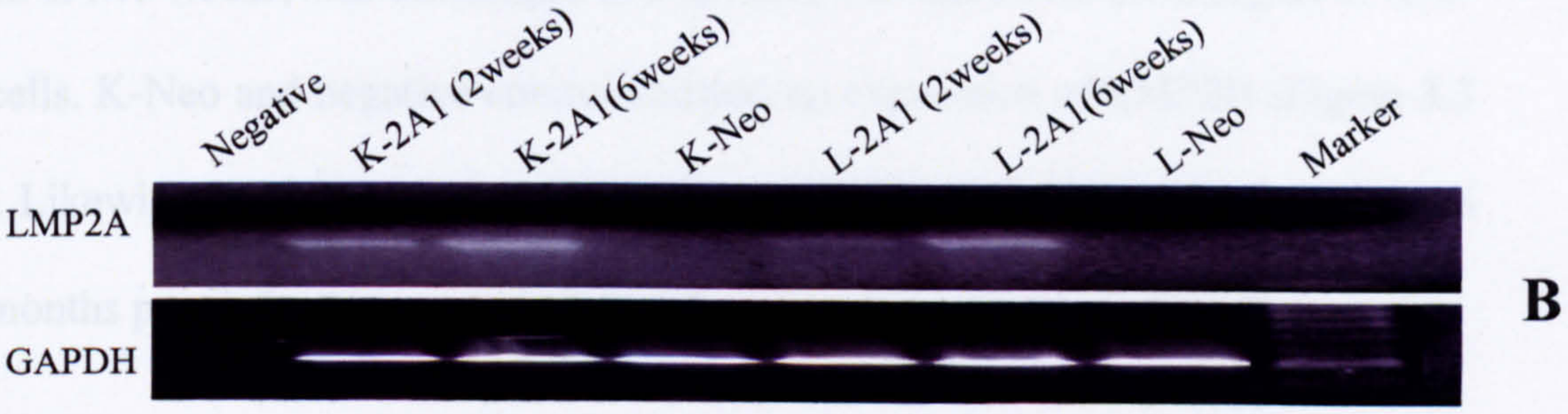
The expression of LMP2 was confirmed by RT-PCR at 6 weeks and 5 months post infection.

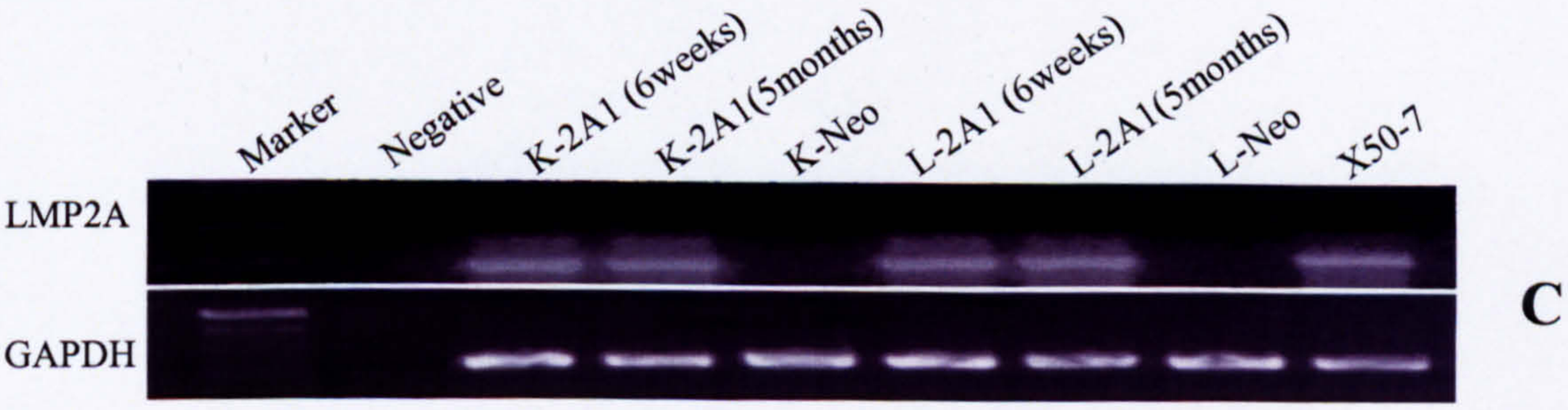
(A) At 6 weeks, the expression of LMP2A in all the 5 clones of KMH2-LMP2A and L428-LMP2A was the same and stronger than at two weeks (K-2A1 at 2 weeks is included as a baseline).

(B) Comparison of the samples K-2A1 and L- 2A1 6 weeks post infection with the corresponding samples 2 weeks post infection, clearly demonstrated this difference (3.2 B).

(C) Furthermore, the expression of LMP2A remained at the same level after 5 months in culture. Thus, after the initial phase, the expression of LMP2A in infected cell lines remains stable over time. RT-PCR for GAPDH was run along side as a loading control.


6weeks
2weeks
Marker
Negative
K-2A1
K-2A2
K-2A3
K-2A4
K-2A5
L-2A1
L-2A2
L-2A3
L-2A4
L-2A5
X50-7
L-Neo
K-2A1
LMP2A
GAPDH
A


Negative
K-2A1 (2weeks)
K-2A1(6weeks)
K-Neo
L-2A1 (2weeks)
L-2A1(6weeks)
L-Neo
Marker
LMP2A
GAPDH
B


Marker
Negative
K-2A1 (6weeks)
K-2A1(5months)
K-Neo
L-2A1 (6weeks)
L-2A1(5months)
L-Neo
X50-7
LMP2A
GAPDH
C

3.2.1.2 Confirmation of the expression of LMP2B in transduced KMH2 and L428 cells

KMH2 and L428 cells expressing LMP2B were generated in the same way as described above for LMP2A. RNA was extracted from 1 clone each of LMP2B transduced KMH2 and L428 cells (K-2B1, L-2B1), 1 clone each of empty vector transduced KMH2 and L428 cells (K-Neo, L-Neo), parental KMH2 and L428 and X50-7 cells. cDNA was made with a specific LMP2B reverse primer. The expression of LMP2B was detectable two weeks post infection in both cell lines, but was weaker than in X50-7 cells (Figure3.3A).

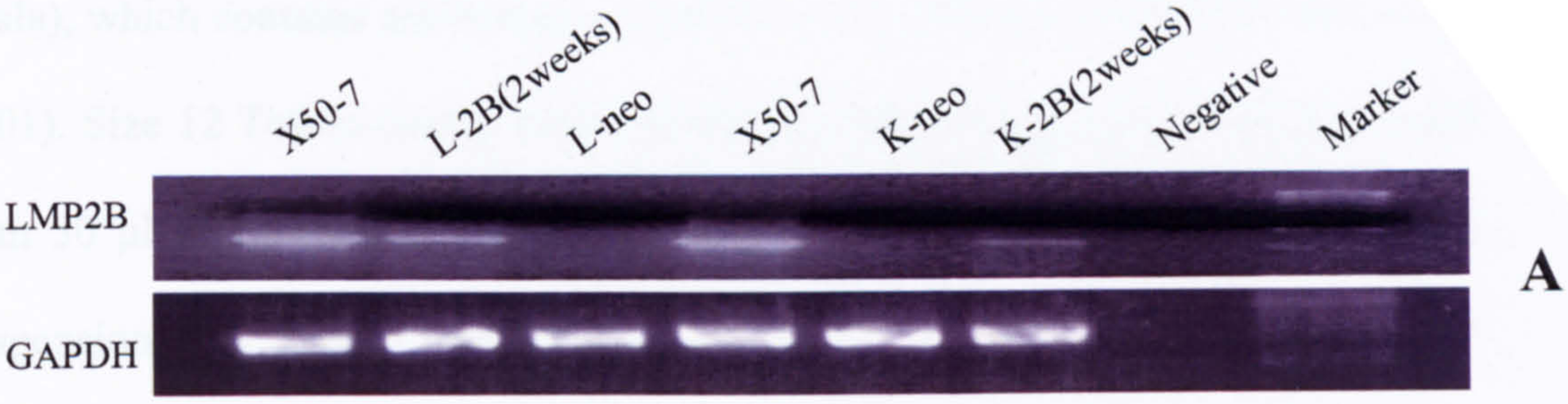
The expression of LMP2B in KMH2 cells 6 weeks post infection was stronger than at two weeks, was unchanged at 5 months, but was never as strong as in X50-7 cells. K-Neo and negative control showed no expression of LMP2B (Figure 3.3 B). Likewise, the expression of LMP2B in L428 cells was stronger at 6 weeks and 5 months post infection compared with 2 week time point (Figure 3.3 C).

Figure 3-3 Expression of LMP2B in transduced KMH2 and L428 cells

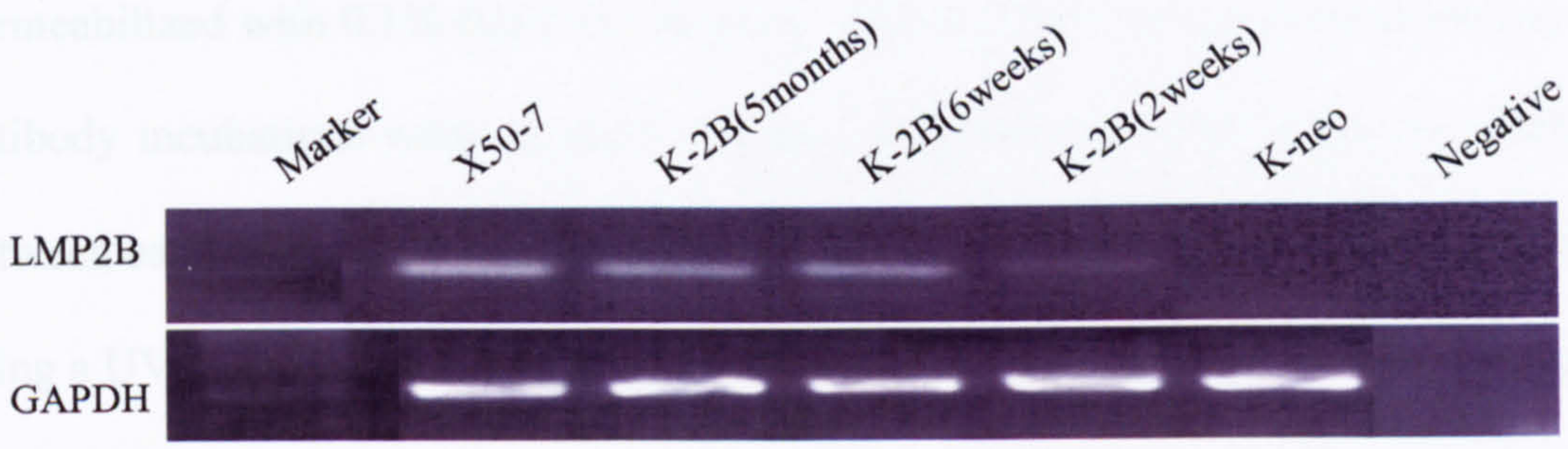
(A)Expression of LMP2B was detectable two weeks post infection in both cell lines, but was weaker than that of X50-7 cells.

(B) The expression of LMP2B in KMH2 cells was increased 6 weeks post infection compared to 2 weeks and was unchanged at 5 months.

(C) Similar results were obtained for expression of LMP2B in L428 cells.


X50-7
L-2B(2weeks)
L-neo
X50-7
K-neo
K-2B(2weeks)
Negative
Marker
LMP2B
GAPDH

A


Marker
X50-7
K-2B(5months)
K-2B(6weeks)
K-2B(2weeks)
K-neo
Negative
LMP2B
GAPDH

B


Marker
X50-7
L-2B(5months)
L-2B(6weeks)
L-2B(2weeks)
L-neo
Negative
LMP2B
GAPDH

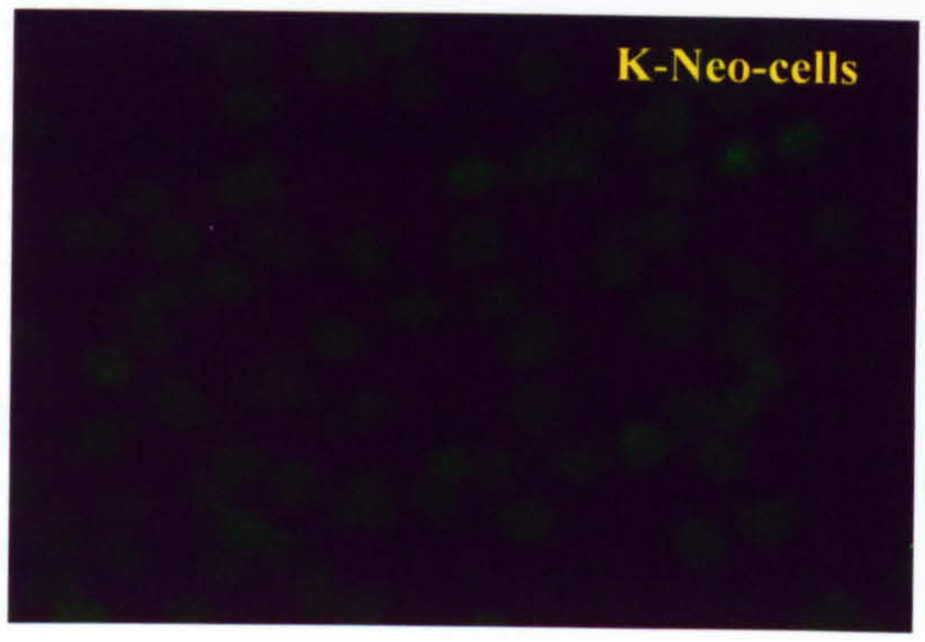
C

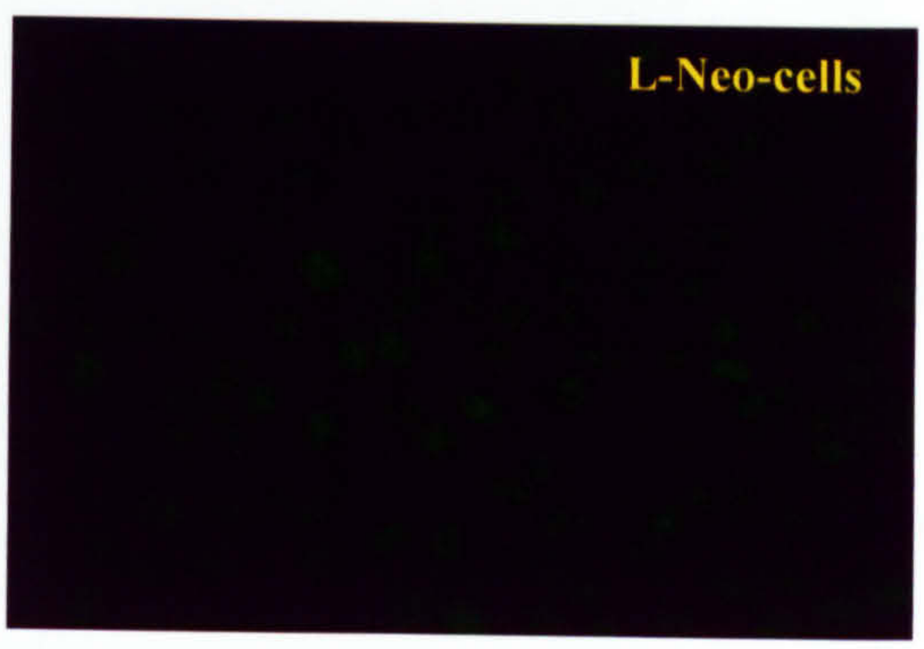
3.2.2 Confirmation of expression of LMP2A and LMP2B protein in retrovirus transduced KMH2 and L428 cells

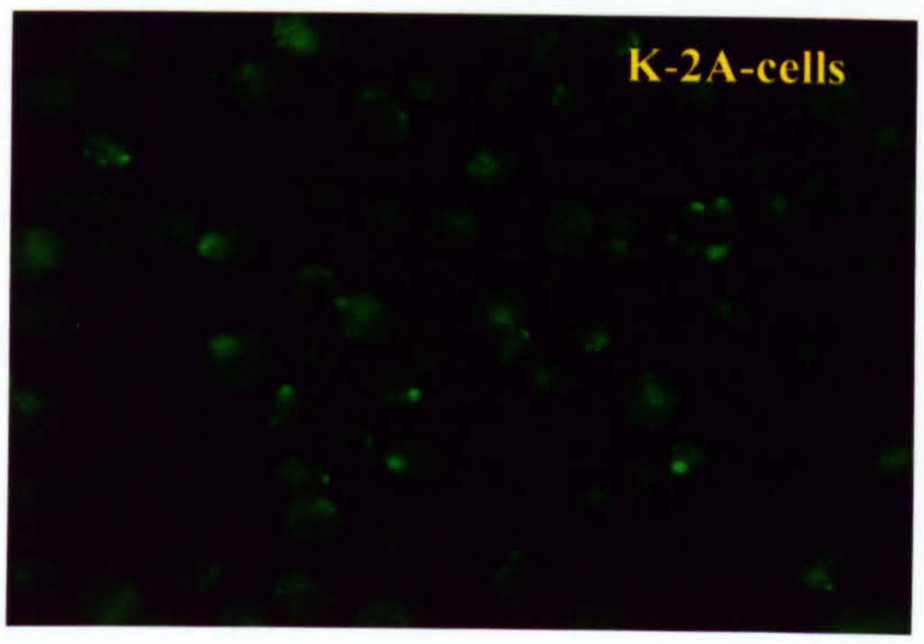
Having determined that the transduced cells expressed LMP2A and LMP2B mRNA, cells were tested for expression of the respective proteins. To do this immunofluorescence staining was performed using serum from an NPC patient (Bala), which contains antibodies to LMP2A and LMP2B proteins (Dawson *et al.*, 2001). Size 12 Teflon-coated slides (Hendley-Essex) were autoclaved and coated with 50 µl of fibronectin in a sterile petridish at 4°C overnight. 50µl of a cell suspension was loaded onto each well of the slide and incubated at 37°C overnight, fixed with 4% PFA (Paraformaldehyde) for 10 minutes and permeabilized with 0.1% (vol/vol) TritonX-100 for 2 min. Primary and secondary antibody incubations were 1h each, and the slides were washed twice with PBS between each of incubations. Coverslips were mounted on glass slides and imaged using a UV microscope. LMP2A transduced KMH2 and L428 cells showed strong granular membrane staining, but the staining of LMP2B was much weaker. Control KMH2-Neo and L428-Neo cells were negative as expected (Figure 3.4).

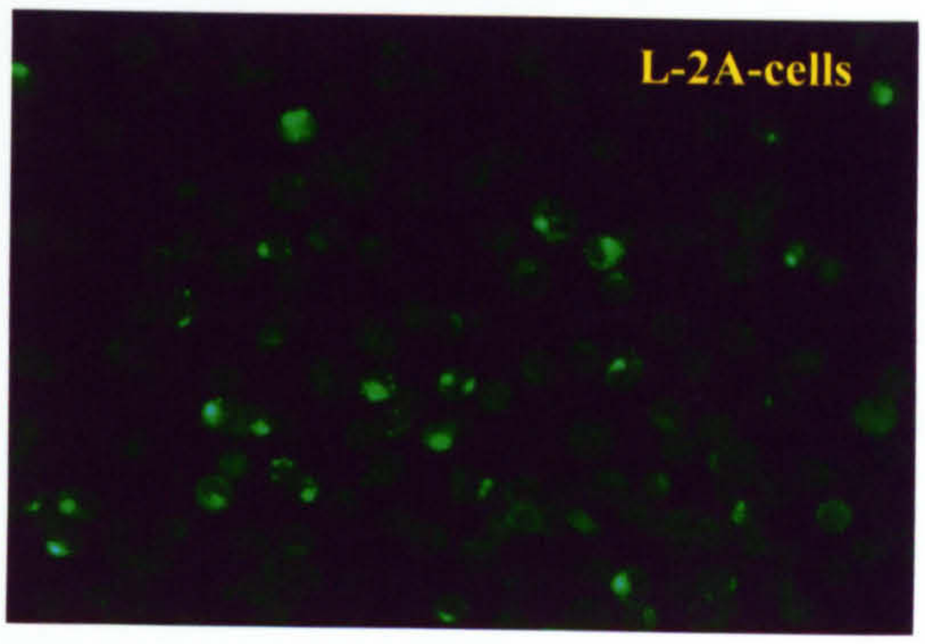
Figure 3-4 Confirmation of expression of LMP2A and LMP2B protein in retrovirus transduced KMH2 and L428 cells

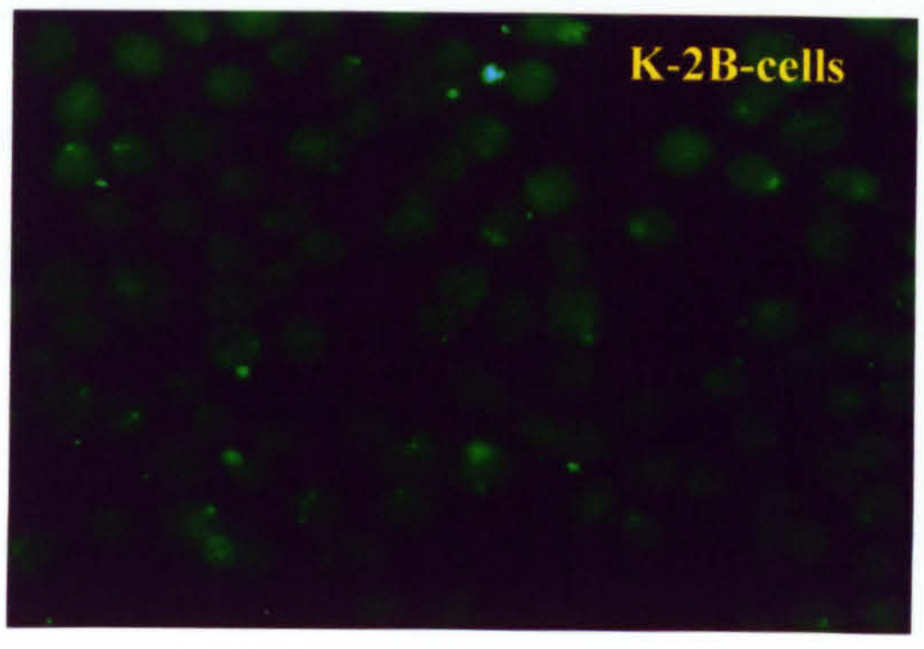
NPC patient serum (Bala) was used as primary antibody (1:50 dilution) and then visualized with Goat-anti-human FITC (1:1000). K-2A and L-2A cells showed bright, granular membrane staining, but the K-2B and L-2B cells were more weakly stained. K-Neo and L-Neo cells were negative.

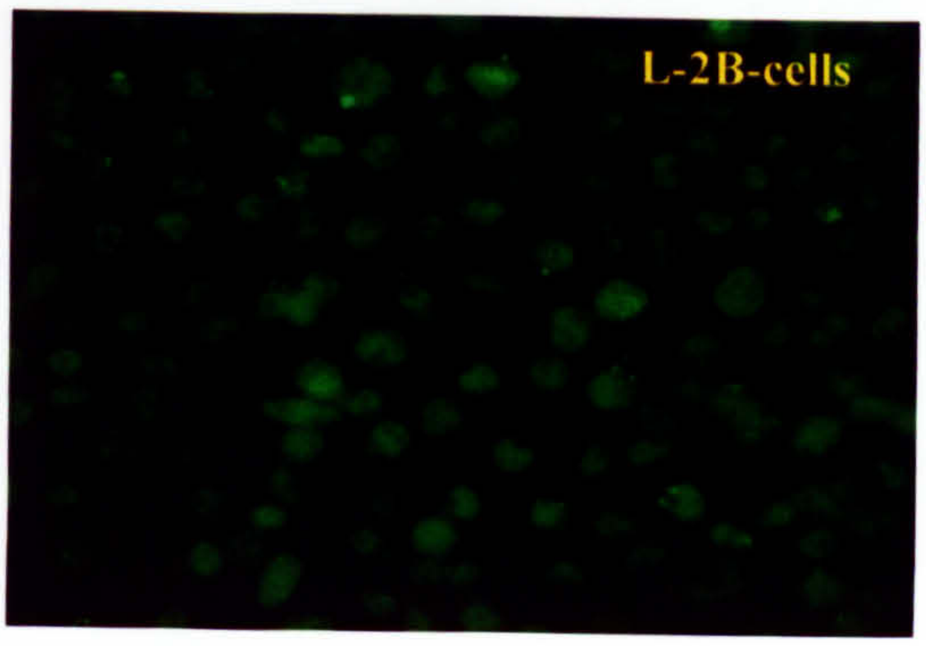

K-Neo-cells


L-Neo-cells


K-2A-cells


L-2A-cells


K-2B-cells


L-2B-cells

3.2.3 Impact of LMP2 expression on the growth and survival of HL cells

3.2.3.1 Proliferation assay

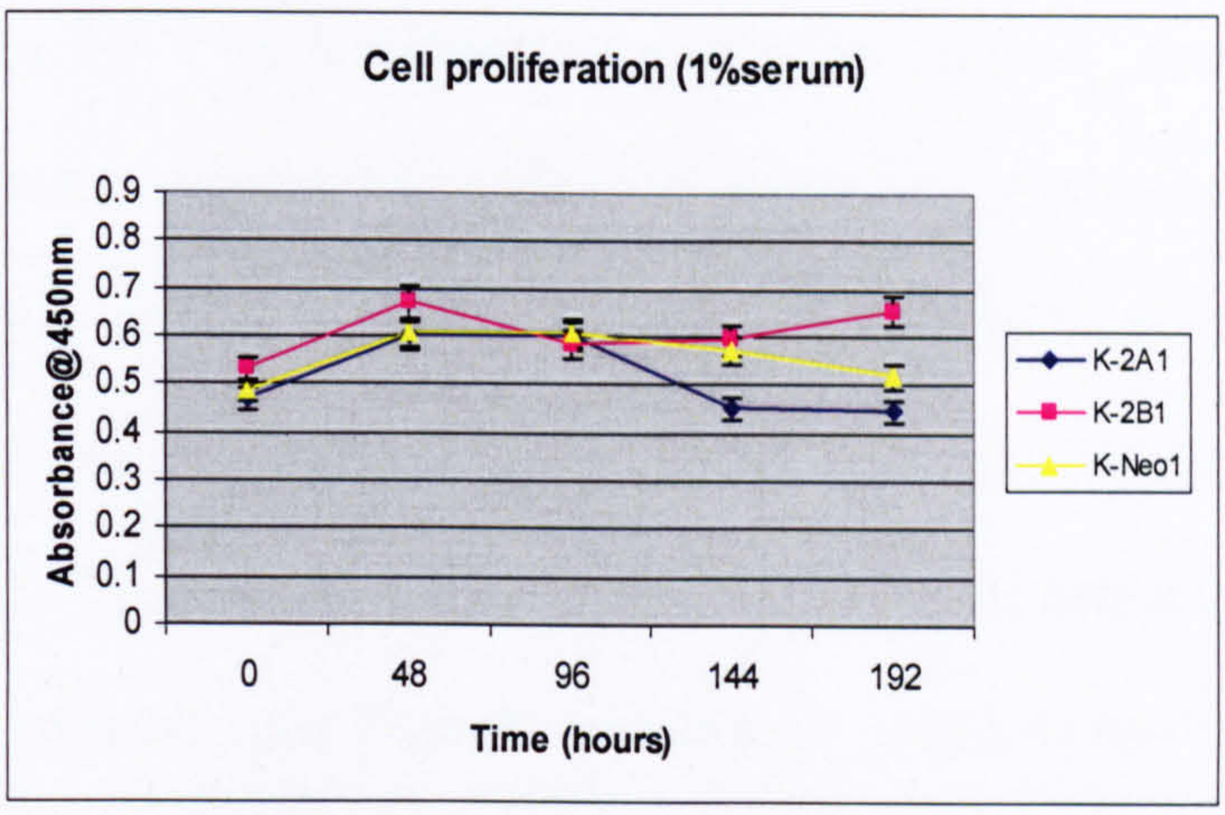
The WST-1 cell proliferation assay was performed on the LMP2A, LMP2B or Neo transduced KMH2 and L428 cells two weeks post infection (See section 2.5.1). 5 x 10^4 Cells from each cell line were washed in serum-free media, resuspended in RPMI medium containing 1%, 5% and 10% B-cell serum (BCS). The WST-1 reagent measures the activity of mitochondrial dehydrogenases in the sample. The tetrazolium salts in the reaction are cleaved to formazan, and the augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates with the number of metabolically active cells in the culture. All quantifiable data were subjected to statistical analysis in Microsoft™ Excel using a two-tailed student's T-test assuming the two samples displayed unequal variance. The p-values indicated a significant difference between the data sets when the p-value was <0.05. The error bars on charts represent the standard error of the mean for each triplicate data set.

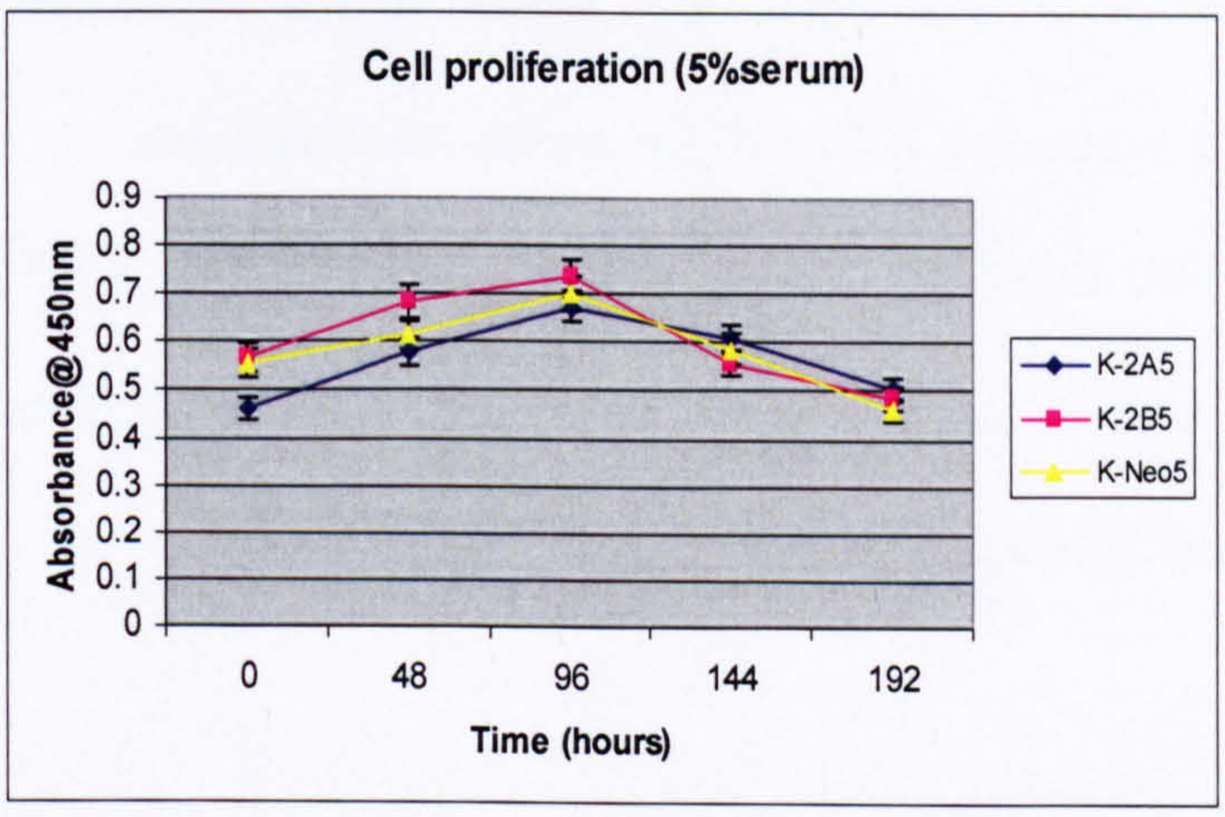
In 10% and 5% serum concentrations LMP2A or LMP2B expressing cells showed very little differences on cell proliferation compared with Neo control cells (Figure 3.5 and 3.6). In 1% serum LMP2A expressing KMH2 cells showed lower cell proliferation at the later time points. This might reflect a toxic effect of high level LMP2A expression in KMH2 cells in low serum.

Figure 3-5 Cell proliferation of K-2A, K-2B and K-Neo expressing cells

5 x 10^4 cells from each cell line were pelleted at 1500 rpm for 5 minutes and resuspended in 5 ml PBS. Cells were washed three times with PBS to remove any trace of serum. Finally, cells were resuspended in 2 ml of growth medium RPMI1640 containing glutamine, penicillin/streptomycin solution and 10%, 5%, or 1% B cell serum without G418 presence. 100 µl of cell suspension from each concentration of serum was pipetted in triplicate to 5 separate 96-well plates. The plates were labelled Day 0, 2, 4, 6 and 8 and incubated at 37° C in 5% CO_2 for up to 8 days without changing the medium. Every 2 days (starting at Day 0), a plate was removed for WST-1 assay. Assessment of cell proliferation was carried out every two days for serum concentration.

When compared under different serum concentration LMP2A or LMP2B expressing in KMH2 cells had little impact on proliferation. The exception was a marginal decrease in LMP2A expressing cells at 144 and 192 hours in 1% serum ($P=0.01$, $p<0.05$). This is possible LMP2 toxicity may predominate over any effect on proliferation. K-2B cells showed a significantly increase in proliferation at 192 hours ($p<0.05$).


Cell proliferation (1%serum)
Absorbance@450nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
48
96
144
192
Time (hours)
K-2A1
K-2B1
K-Neo1


Cell proliferation (5%serum)
Absorbance@450nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
48
96
144
192
Time (hours)
K-2A5
K-2B5
K-Neo5

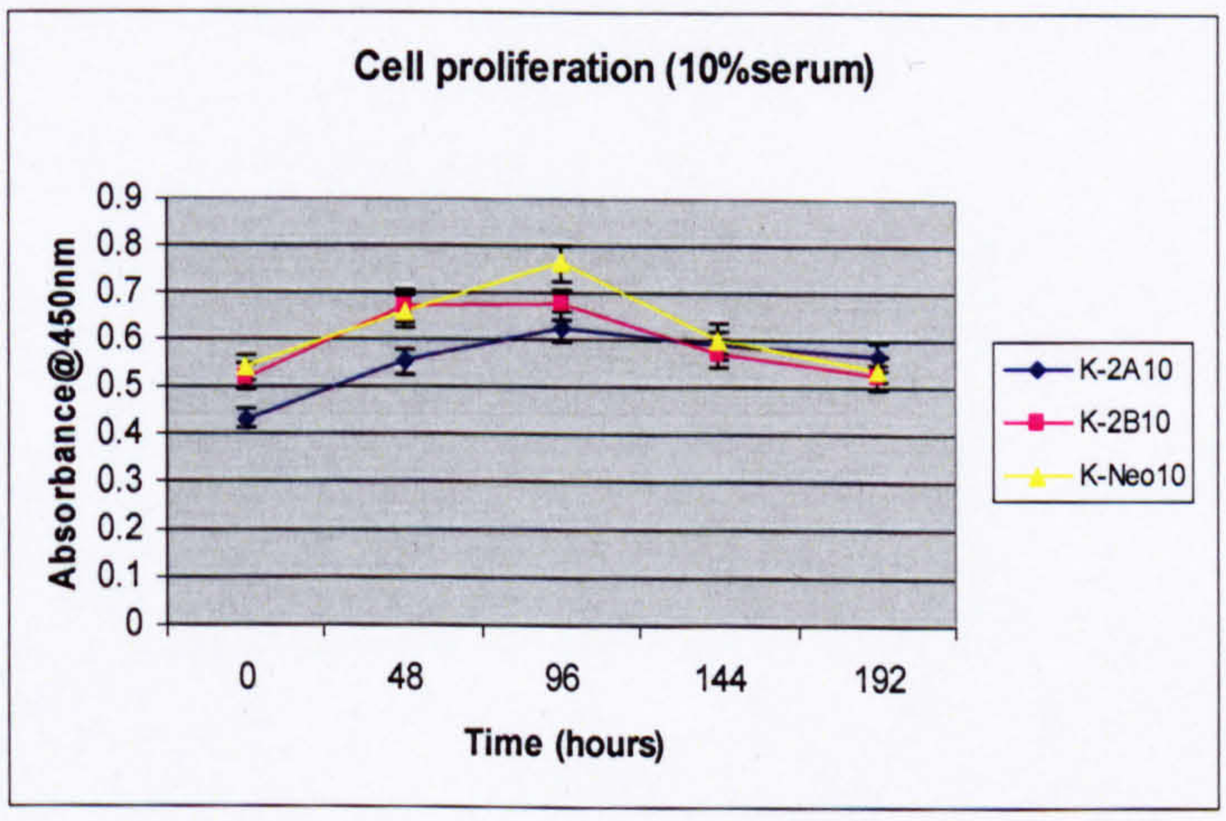
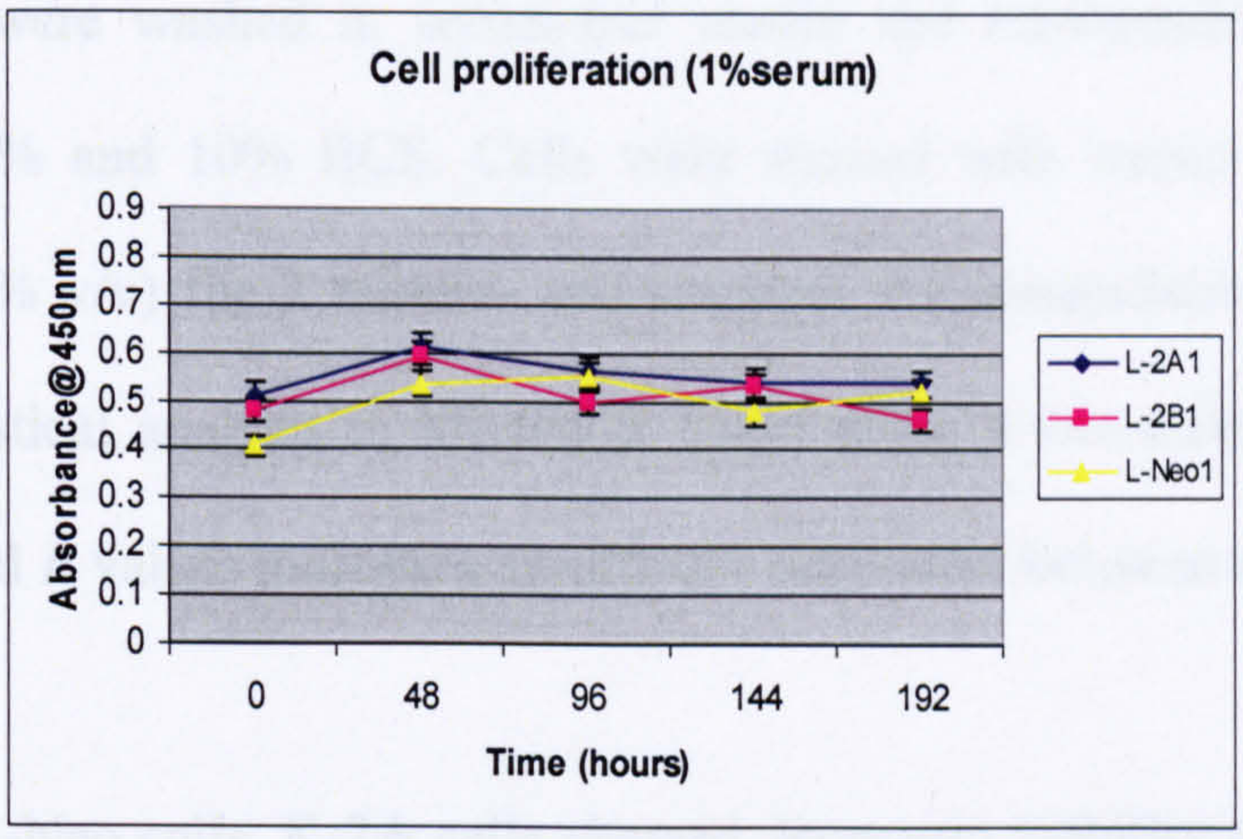
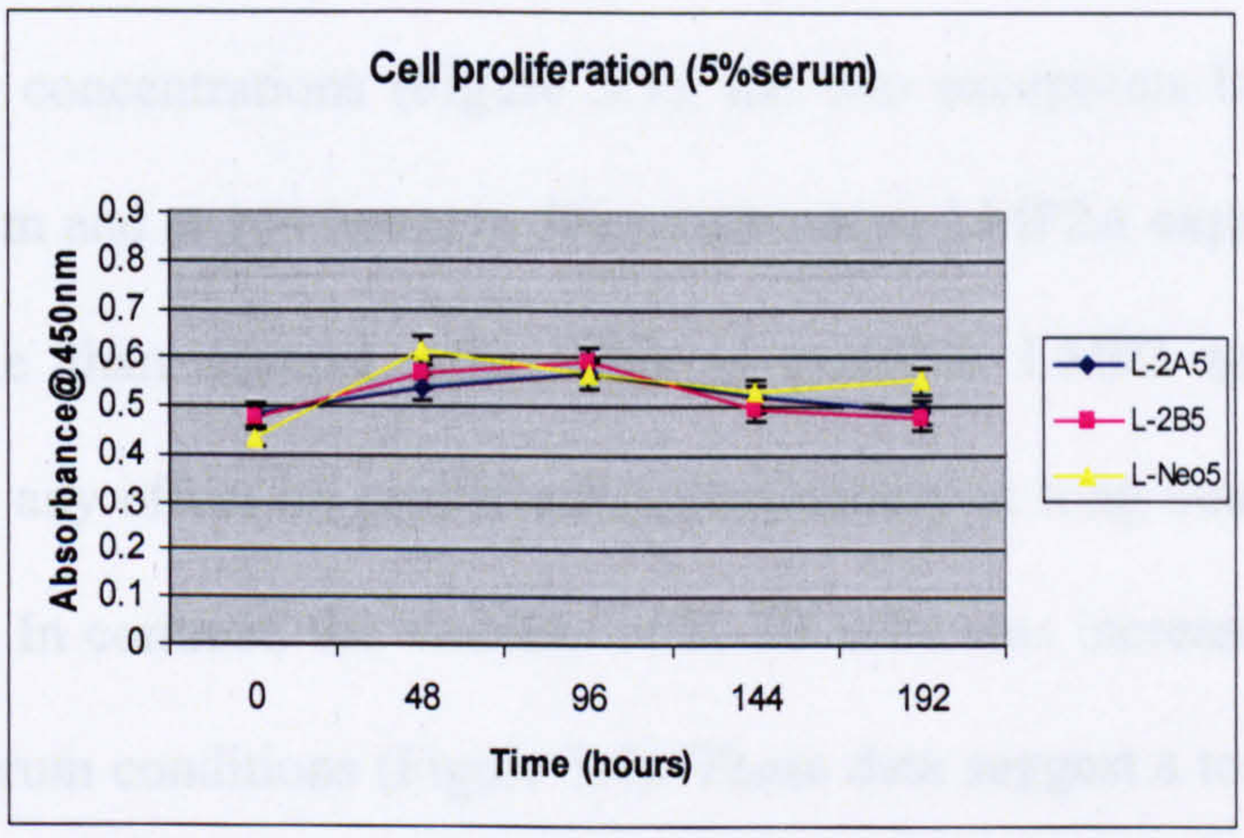

Cell proliferation (10%serum)
Absorbance@450nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
48
96
144
192
Time (hours)
K-2A10
K-2B10
K-Neo10

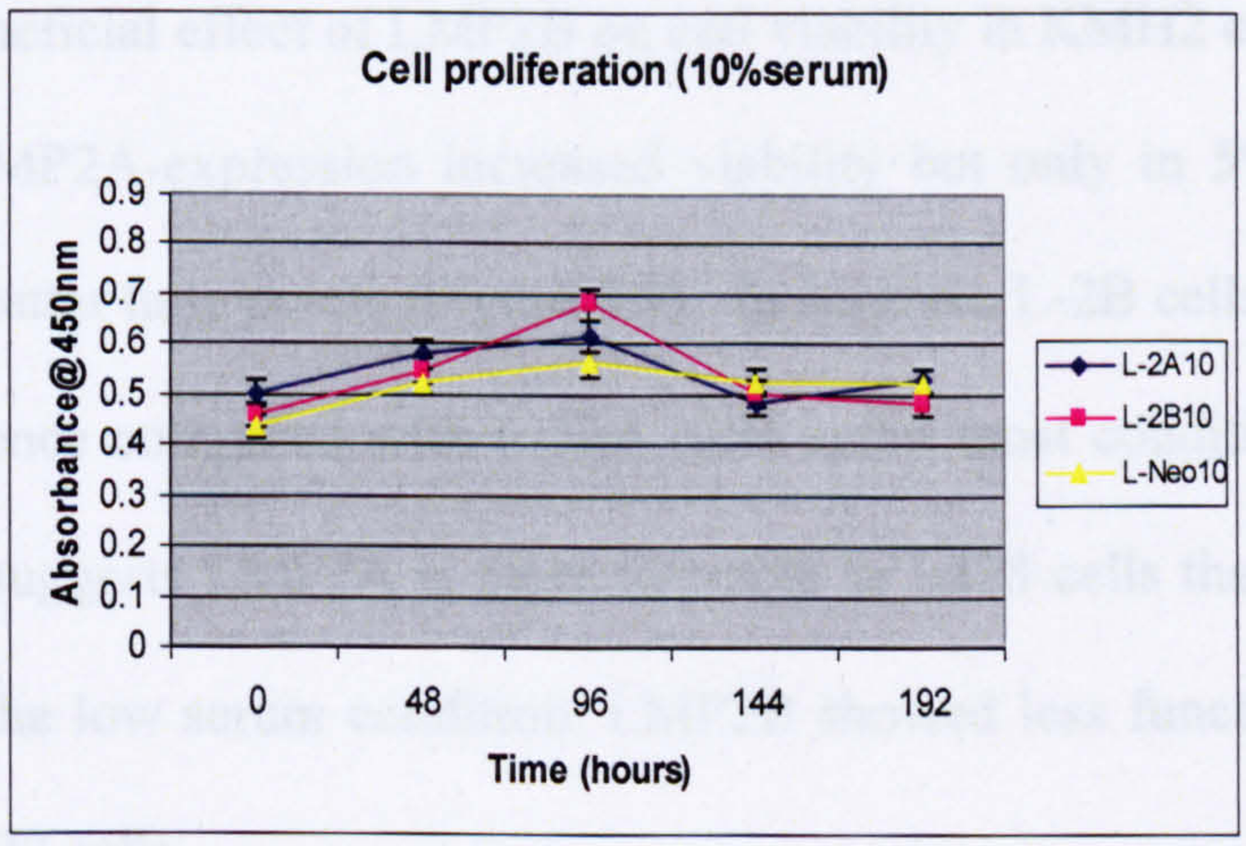
Figure 3-6 Proliferation of L-2A, L-2B and L-Neo expressing cells

5 x 10^4 cells from each cell line were pelleted at 1500 rpm for 5 minutes and resuspended in 5 ml PBS. Cells were washed three times with PBS to remove any trace of serum. Finally, cells were resuspended in 2 ml of growth medium RPMI1640 containing glutamine, penicillin/streptomycin solution and 10%, 5%, or 1% B cell serum without G418 presence. 100 µl of cell suspension from each concentration of serum was pipetted in triplicate to 5 separate 96-well plates. The plates were labelled Day 0, 2, 4, 6 and 8 and incubated at 37° C in 5% CO_2 for up to 8 days without changing the medium. Every 2 days (starting at Day 0), a plate was removed for WST-1 assay. Assessment of cell proliferation was carried out every two days (starting at Day 0) for serum concentration.

Compared with L-Neo cells, expression of LMP2A or LMP2B in L428 cells showed no significant difference in proliferation under three different serum conditions and 5 time points.


Cell proliferation (1%serum)
Absorbance@450nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
48
96
144
192
Time (hours)
L-2A1
L-2B1
L-Neo1


Cell proliferation (5%serum)
Absorbance@450nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
48
96
144
192
Time (hours)
L-2A5
L-2B5
L-Neo5


Cell proliferation (10%serum)
Absorbance@450nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
48
96
144
192
Time (hours)
L-2A10
L-2B10
L-Neo10

3.2.3.2 Survival assay

Trypan blue exclusion assays were performed on LMP2A, LMP-2B and Neo transduced KMH2 and L428 cells two weeks post transfection (see Section 2.5.2). 1.2 x 10^6 Cells were washed in serum-free media and resuspended in RPMI containing 1%, 5% and 10% BCS. Cells were stained with trypan blue (final concentration, 20% v/v) for 2 minutes and counted. All quantifiable data were subjected to statistical analysis in Microsoft Excel using a two-tailed Student *t* test. The generated *P* values indicate a significant difference between the data sets when $P < 0.05$.

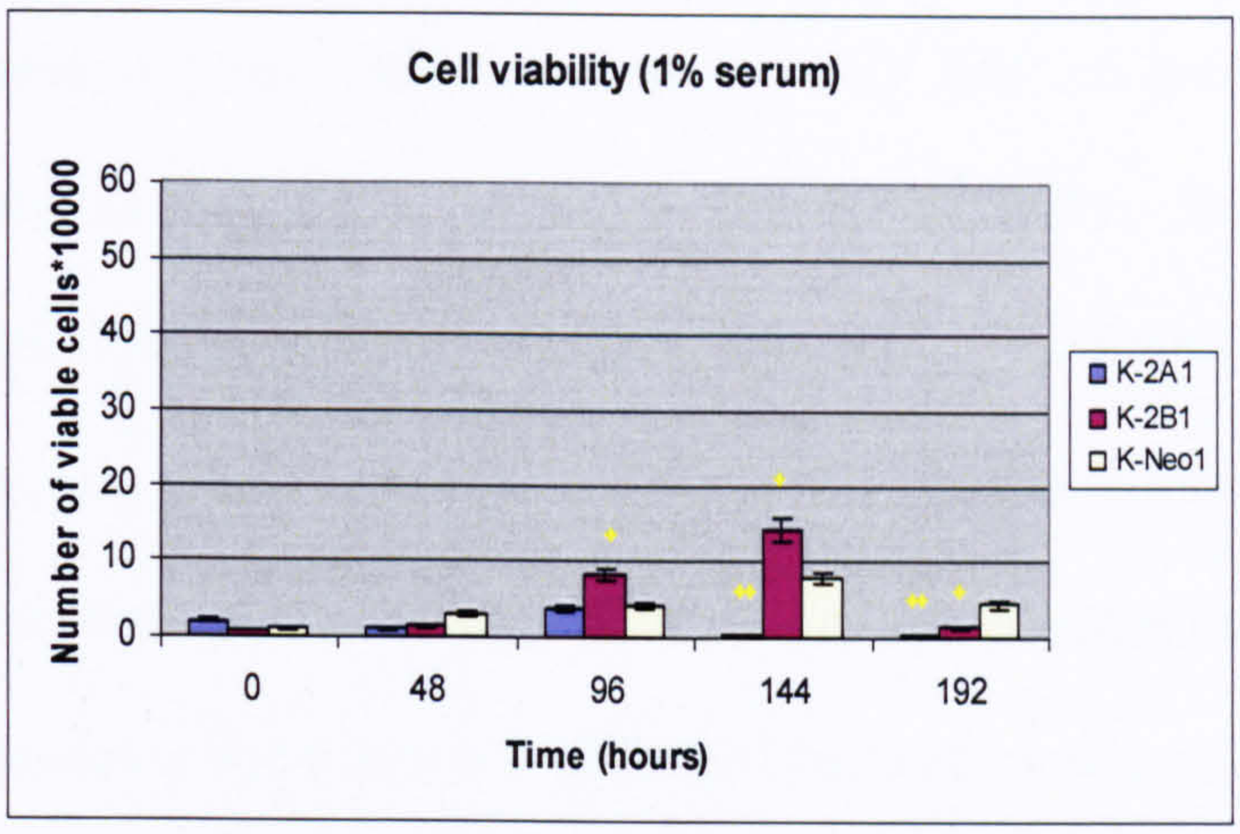
Compared with K-Neo cells, K-2A cells showed decreased viability at most time points and serum concentrations (Figure 3.7), the two exceptions being at 192 hours in 10% serum and at 144 hours in 5% serum where LMP2A expressing cells were more viable than control cells. This is possible LMP2 toxicity may predominate over any effect on proliferation, particularly at long hours with low serum condition. In contrast, the viability of K-2B cells was increased up to144 hours under all serum conditions (Figure 3.7). These data suggest a toxic effect of LMP2A and a beneficial effect of LMP2B on cell viability in KMH2 cells.

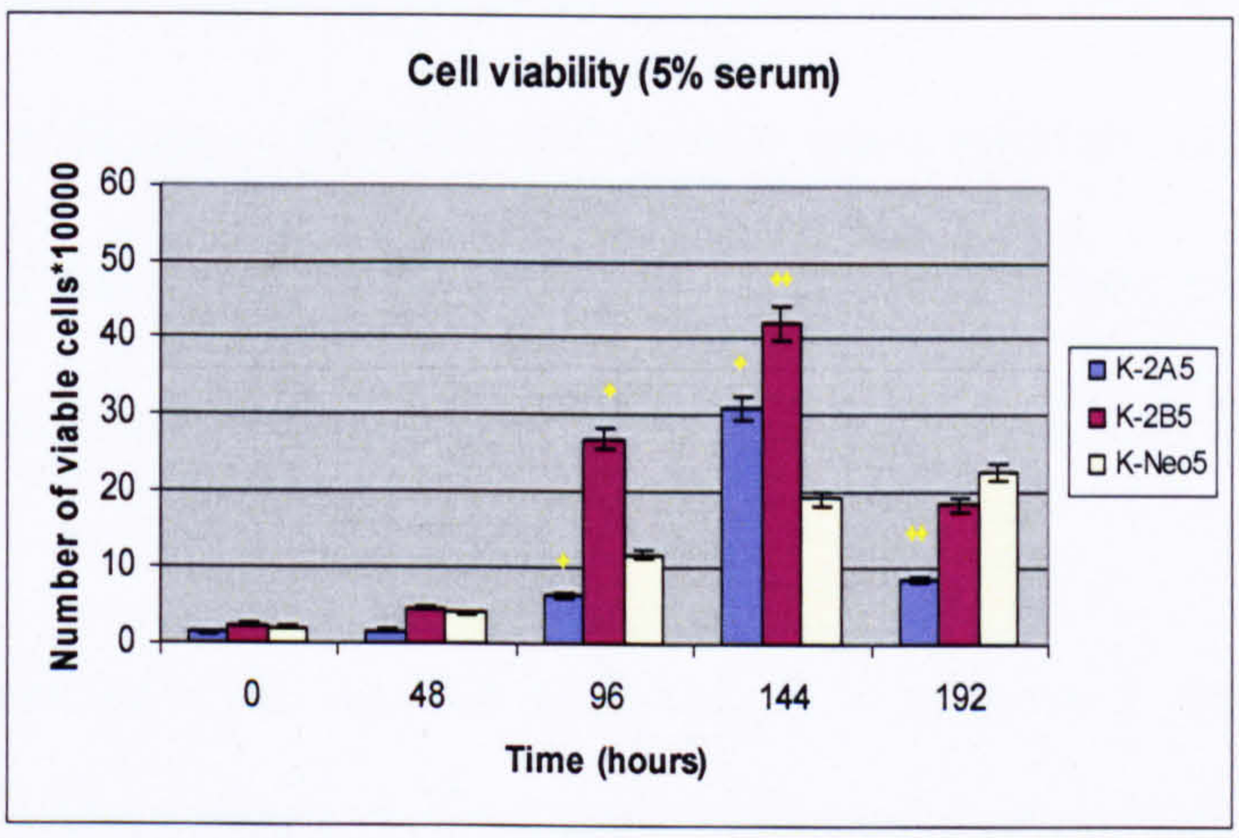
In L428 cells, LMP2A-expression increased viability but only in 5% and 10% serum and at the latter time points (Figure 3.8). In contrast, L-2B cells showed no significant difference compared with L-Neo cells under most conditions (Figure 3.8). This result suggests LMP2A is more tolerable in L428 cells than in KMH2 cells, even if in the low serum condition. LMP2B showed less function in L428 cells than in KMH2 cells.

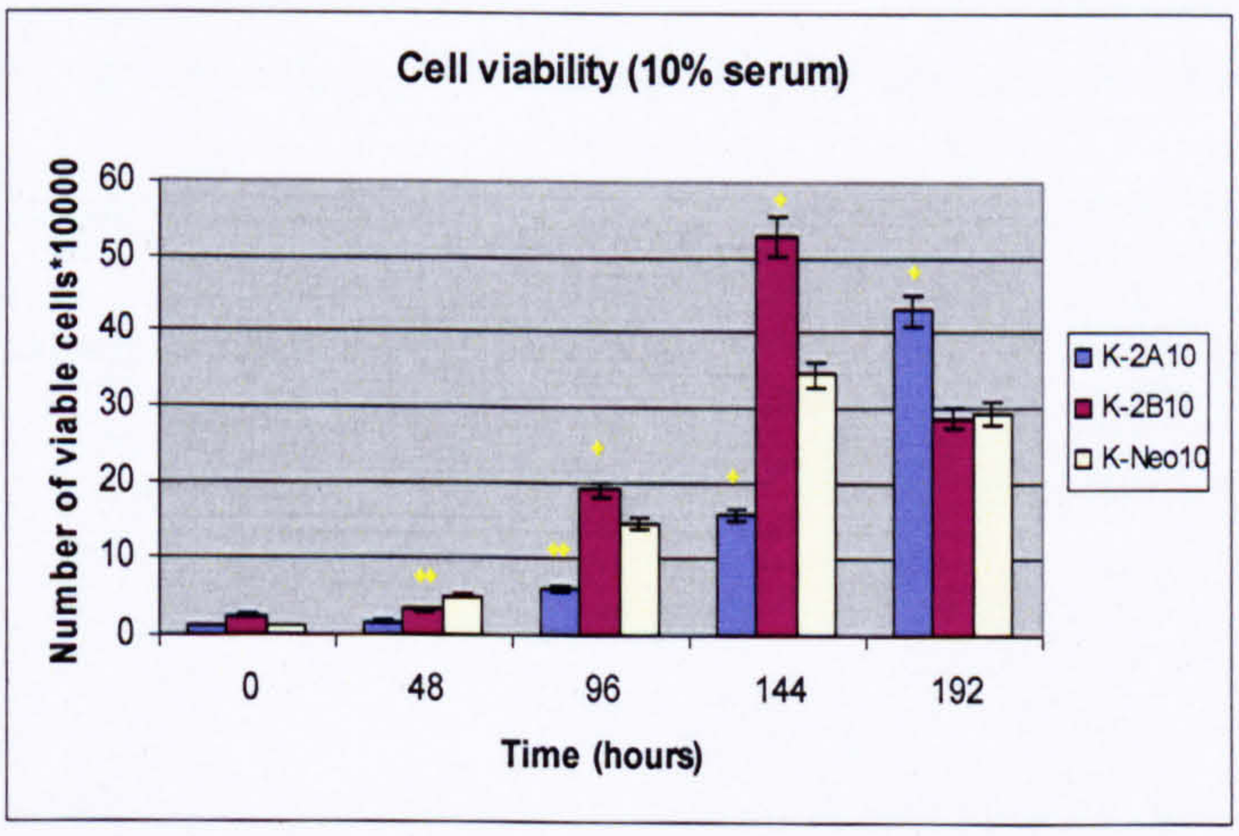
Figure 3-7 Viability of K-2A, K-2B and K-Neo expressing cells

1.2×10^6 cells from each cell line were pelleted at 1500 rpm for 5 minutes and resuspended in 5 ml PBS. Cells were washed three times to remove any trace of serum. Finally, cells were resuspended in 4 ml cell maintenance medium, RPMI1640 containing glutamine, penicillin/streptomycin solution and 10%, 5%, or 1% B cell serum without G418 presence. 200 µl of each cell suspension at each of the different serum concentrations was pipetted in triplicate into 5 separate 48-well plates. Plates were labelled Day 0, 2, 4, 6 and 8 and incubated at 37° C in 5% CO_2 for up to 8 days without changing the medium. Every 2 days (starting at Day 0), a trypan blue viability assay was carried out with trypan blue reagent. 80 µl of cell suspension was mixed with 20 µl trypan blue reagent for 2 minutes and the cell viability was evaluated by direct counting of unstained cells under a microscope.

Compared with K-Neo cells, K-2A cells showed decreased viability at most time points and three serum concentrations; the exception being at 192 hours in 10% serum and at 144 hours in 5% serum where LMP2A expressing cells showed increased viability compared with K-Neo cells. In contrast, the viability of K-2B cells was usually higher than K-Neo cells. All results are expressed as the number of viable cells times 10 thousands at each time point, and represent the mean of 3 independent experiments.







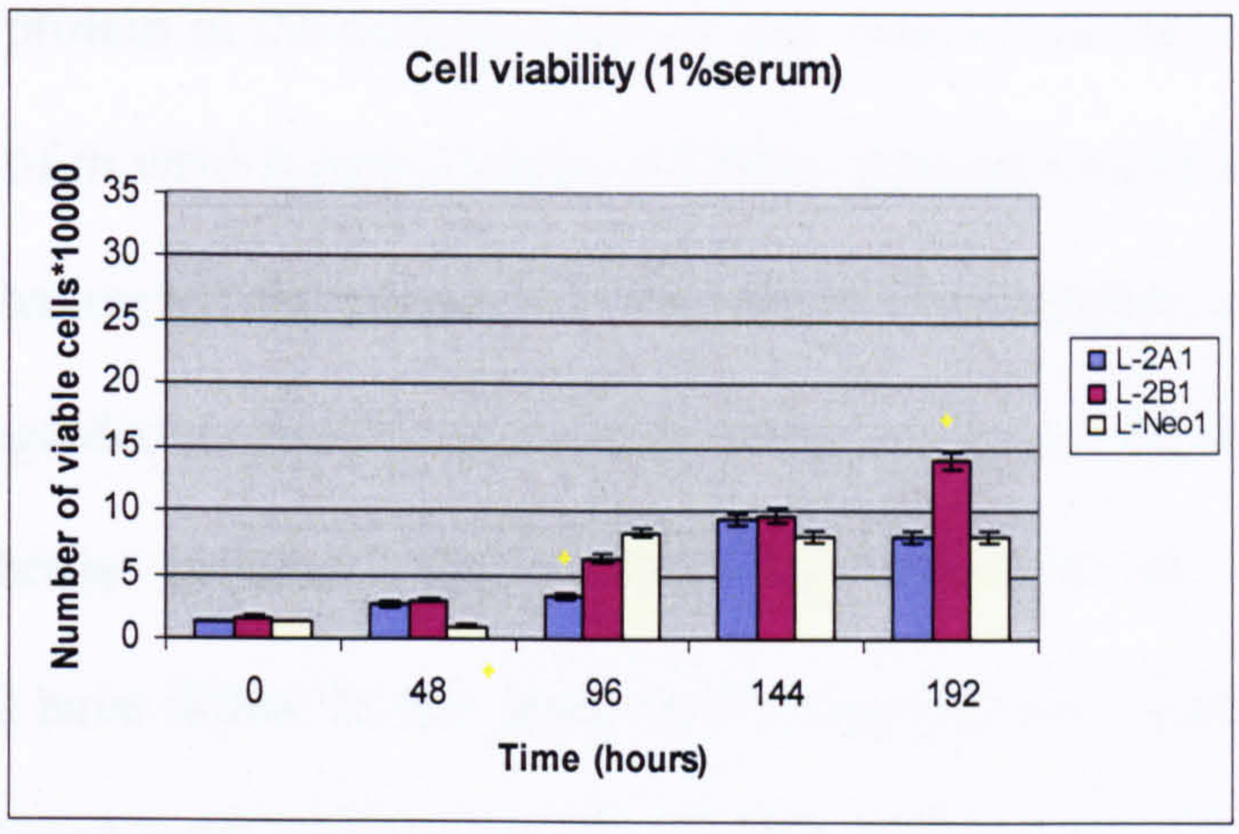
Denote: ✦✦ P<0.01

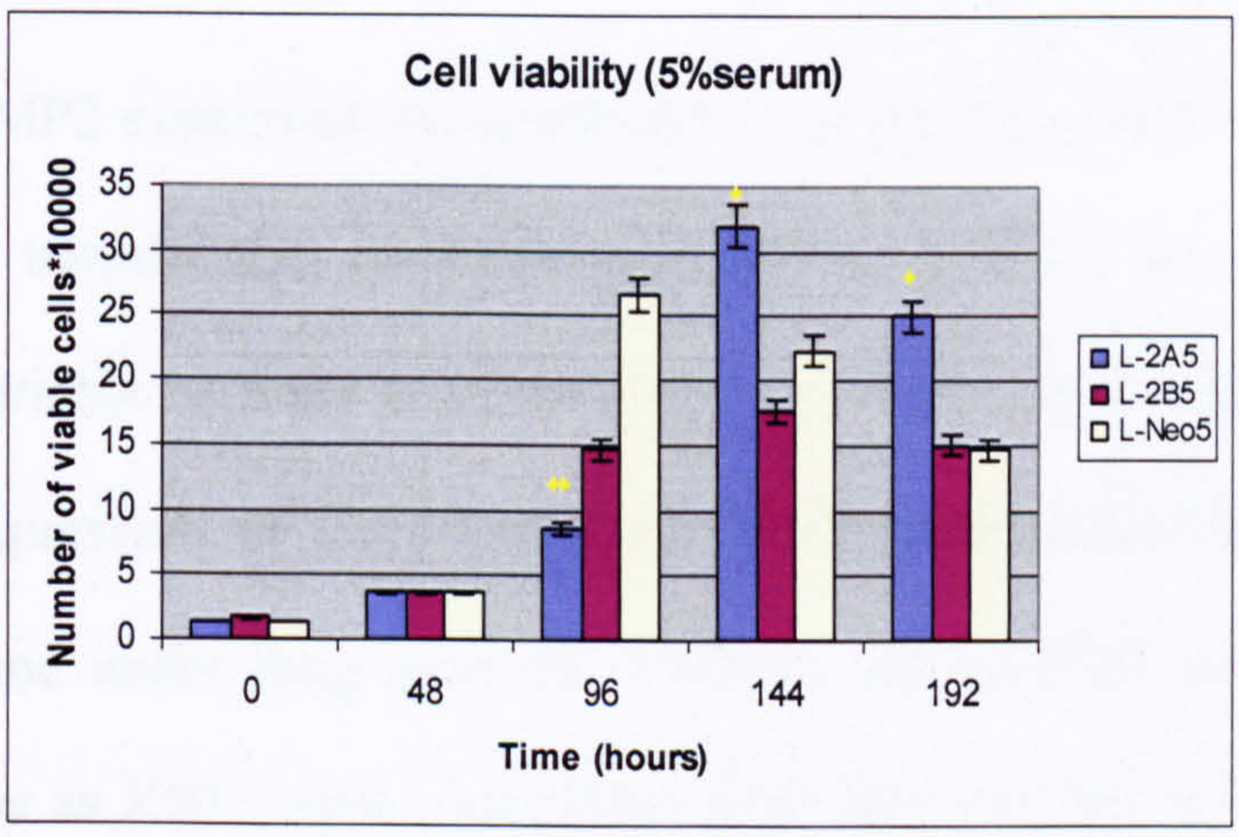
✦ P<0.05

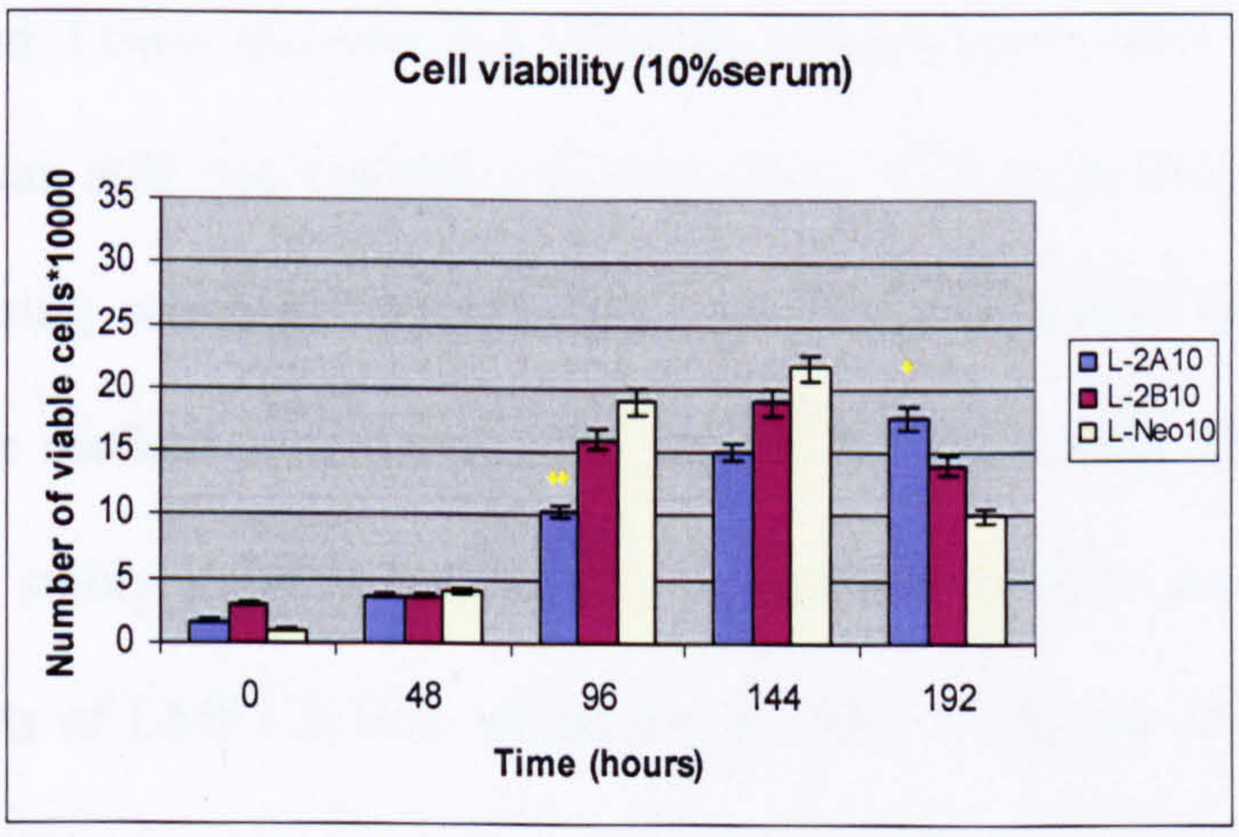
Figure 3-8 Viability of L-2A, L-2B and L-Neo expressing cells

1.2 x 10^6 cells from each cell line were pelleted at 1500 rpm for 5 minutes and resuspended in 5 ml PBS. Cells were washed three times to remove any trace of serum. Finally, cells were resuspended in 4 ml cell maintenance medium, RPMI1640 containing glutamine, penicillin/streptomycin solution and 10%, 5%, or 1% B cell serum without G418 presence. 200 µl of each cell suspension at each of the different serum concentrations was pipetted in triplicate into 5 separate 48-well plates. Plates were labelled Day 0, 2, 4, 6 and 8 and incubated at 37° C in 5% CO_2 for up to 8 days without changing the medium. Every 2 days (starting at Day 0), a trypan blue viability assay was carried out with trypan blue reagent. 80 µl of cell suspension was mixed with 20 µl trypan blue reagent for 2 minutes and the cell viability was evaluated by direct counting of unstained cells under a microscope.

Compared with L-Neo cells, L-2A cells showed no significant difference in viability in 1% serum; however, viability of LMP2A-expressing cells was increased at 144 hours and 192 hours in 5% serum and at 192 hours in 10% serum. Overall, viability of L-2B cells was not different to that of L-Neo cells. All results are expressed as the number of viable cells times 10 thousands at each time point, and represent the mean of 3 independent experiments.







Denote: ++ P<0.01

+ P<0.05

3.3 Discussion

The consistent expression of LMP2A in primary HL suggests an important function for this protein in the pathogenesis of this tumour but this remains to be shown. The lack of *in vitro* systems whereby LMP2 is expressed in a relevant cell background has hampered the research in this field. The transfection of HL cell lines is always regarded as problematic and possibly more so if the transgenes are toxic transmembrane proteins. Many efforts to establish HL cells stably expressing LMP2 have failed. In this study stable expression of both LMP2A and LMP2B was achieved in HL cells using retrovirus infection.

The pattern of LMP2 expression documented here is similar in both HL cell lines. Two weeks post transduction, the expression of both LMP2A and LMP2B was detectable but variable between different clones. However, at 6 weeks with drug selection, the expression of the LMP2A and LMP2B increased and remained constant over time under drug pressure. LMP2A and LMP2B expression was lower or the same as X50-7 cells suggesting "physiological" levels of expression has been achieved. Lower expression soon after infection may have been because drug selection was still not complete at that time. This work demonstrates the advantages of using retroviral transduction over standard DNA transfection to provide a reliable method to transduce HL cell lines with LMP2. The generation of HL cell lines stably expressing LMP2A or LMP2B enables the study of the phenotypic effects of LMP2 in this environment. This is the first time that stable LMP2A or LMP2B expression has been achieved in EBV-negative HL cells.

LMP2A activates PI3-K/Akt pathway, which acts as a cell survival signal in B-cell lines (Swart *et al.*, 2000). LMP2A also appears to be a key determinant in the alteration of epithelial cell growth by activating Akt and c-Jun and leading to

enhancement of cell growth (Chen *et al.*, 1999; Scholle *et al.*, 2000). In addition, LMP2A expression is important in epithelial cell clone outgrowth following infection of epithelial cells (Moody *et al.*, 2003). Another report showed that LMP2A mediated the survival of primary, BCR-negative B cells by constitutive activation of the Ras/PI3K/Akt pathway (Portis and Longnecker, 2004). EBV-infected KMH2 cells, displaying a pattern of virus gene expression largely restricted to Qp-driven EBNA1, LMP2, EBERs, and the *Bam*H1A transcripts, showed significantly increased cell growth and survival compared with uninfected controls (Baumforth *et al.*, 2005). Thus, LMP2 might play an important role in inducing the growth and survival of EBV-positive HRS cells. Interestingly, the LMP2 transduced HL cell lines failed to show any clear increase in proliferation compared with control cells. There are a number of possible explanations: First, the assays were performed 2 weeks post infection; at this time cells were still in the initial stages of selection. Second, it is possible LMP2 toxicity may predominate over any effect on proliferation, particularly it was shown at, 144 and 192 hours with low serum medium. This problem may be overcome by changing the medium during the assay. Third, the effects of LMP2 might also depend on levels of expression; whether higher levels of LMP2 expression induces the cell proliferation remains to be investigated. The effects of LMP2A and LMP2B on survival were also not consistent and varied between the two cell lines; this might reflect their different sensitivity to the toxic effect of LMP2A or LMP2B protein; LMP2A approved to be better tolerated by L428 cell than KMH2 cells.

In conclusion, this study has generated cell line models that enable the influence of LMP2A and LMP2B expression to be assessed in an HL cell background. The failure to identify any clear major phenotype effect of either protein in HL cells

might suggest more subtle effect of these two viral genes in the pathogenesis of HL.

CHAPTER FOUR: ANALYSIS OF THE INFLUENCE OF LMP2 EXPRESSION ON THE CELLULAR TRANSCRIPTIONAL PROGRAMME OF HODGKIN'S LYMPHOMA CELLS

4 Analysis of the influence of LMP2 expression on the cellular transcriptional programme of Hodgkin's lymphoma cells

4.1 Introduction

Classical HL is characterized by the presence of usually less than 1% of HRS cells in the tumour tissues. HRS cells show a peculiar phenotype that includes downregulation of B cell markers and expression of markers of other hematopoietic lineages (Kuppers, 2002, kuppers 2004). About 50% of HL are associated with EBV, and LMP2 is consistently expressed. Expression of LMP2A in transgenic mice alters B-cell development by inhibiting Ig heavy-chain gene rearrangement and expression of a functional BCR (Caldwell *et al.*, 2000; Caldwell *et al.*, 1998). This suggests that LMP2A may alter the expression and/or activity of transcription factors or signalling molecules required for proper B-cell development. However, the exact function of LMP2 in the pathogenesis of HL remains unknown. To obtain further insight into the role of LMP2A in the pathogenesis of HL, a global gene expression analysis was carried out on LMP2-expressing HL cells using microarray analysis.

The development of microarray analysis has provided a powerful tool to study altered gene expression patterns in biological systems. Recently, DNA microarray technology performed with LMP2A transgenic mice, LMP2A-expressing human B-cell lines, and LMP2A-positive and -negative EBV-infected lymphoblastoid cell lines (LCLs) demonstrated that LMP2A increased the expression of genes associated with cell cycle induction and inhibition of apoptosis. LMP2A also altered the expression of genes involved in DNA and RNA metabolism, and decreased the expression of B-cell-specific factors and genes associated with

immunity. Indeed, many alterations in gene expression induced by LMP2A were similar to those recently described in HRS cells of Hodgkin's lymphoma and activated, proliferating germinal centre centroblasts/centrocytes (Portis and Longnecker, 2003; Portis *et al.*, 2003). To date, there has been no comprehensive study of the impact of LMP2 expression in HL cells. In our laboratory, EBV negative KMH2 cells have already been stably infected with recombinant Akata-derived EBV (Baumforth *et al.*, 2005) and subjected to global gene expression analysis when compared to their EBV-negative parental counterparts. In this study, microarray analysis was performed on the KMH2 cells described in chapter 3 which stably expressed either LMP2A or LMP2B.

Aims of this study:

1. Detection of global gene expression alterations in KMH2 cells expressing LMP2A or LMP2B through the use of gene expression microarrays.
2. Validation of the selected gene expression changes observed by semi-Q-PCR

4.2 Results

4.2.1 Gene expression array analysis of the contribution of LMP2 to cellular gene transcription in the HL-derived cell line, KMH2

Affymetrix gene expression arrays were used to investigate the effect of LMP2 expression in KMH2 cells. RNA was extracted from all three cell lines, used to make cDNA, cleaned up, converted to antisense RNA by *in vitro* transcription, fragmented and hybridised to an Affymetrix Human U133 Plus 2 arrays according to the methods detailed in section 2.6. Retrovirus carrying LMP2A, LMP2B and control Neo gene infected KMH2 cells were used (Chapter3). Clone K–2A1 was selected to carry out the array assay because the expression level of LMP2A is close to its physiological level. Too high expression of LMP2A possibly results in false results and low expression of LMP2A wouldn't cause cellular gene alteration. Each experiment was performed in triplicate using RNA from separate flasks of cells, one flask of which were 2 weeks post infection (2AI, 2BI, NeoI) and another two flasks of which were 8 weeks post infection (2AII, 2AIII, 2BII, 2BIII, NeoII, NeoIII). The arrays were scanned and analysed using RAM and rank products method as described in section 2.6.10, to identify those genes significantly up or down regulated in the presence of either LMP2A or LMP2B expression. Differentially expressed genes were then clustered in a "heatmap" using dChip where their relative expression intensities were compared. In the "heatmap" for each gene, white represents the mean expression level of the three biological replicates, increasing intensity of red or blue represent increased or decreased expression relative to the mean in LMP2A or LMP2B expressing cells compared to control cells. Gene changes were deemed significant when the percentage of false-positives (pfp) was <10%.

197 genes were significantly altered by LMP2A, including 143 up regulated genes (Figure 4.1 A) and 54 down regulated genes (Figure 4.1 B).

There were 49 genes significantly changed in the presence of LMP2B in KMH2 cells, including 47 up regulated genes and 2 down regulated genes (Figure 4.2). 49% of genes altered by LMP2B were also affected by LMP2A (23 upregulated and 1 downregulated) (Figure 4.3).

It was clear from the heatmap that the expression level of a significant number of genes was influenced by the time post-infection at which the cells were harvested. Thus, the first replicate for LMP2A, LMP2B or Neo expressing cells was harvested at 2 weeks, whereas the two others were taken at 8 weeks. Since it was previously shown that LMP2A or LMP2B expression increased at 8 weeks compared to 2 weeks, it is possible that these differences in cellular gene expression are a consequence of the differing levels of each protein.

Figure 4-1 Heatmap of microarray data comparing cellular gene expression between LMP2A-expressing KMH2 cells and vector only control cells

Colour intensities represent the mean expression level of the three biological replicates; increasing intensity of red or blue represent increased or decreased expression relative to the mean (white) in LMP2A-expressing KMH2 cells compared to vector only control cells. Clone K–2A1 was selected to carry out the array assay because the expression level of LMP2A is close to its physiological level and low expression of LMP2A wouldn't cause cellular gene alteration. Each experiment was performed in triplicate using RNA from separate flasks of cells, one flask of which were 2 weeks post infection (2AI and NeoI) and another two flasks of which were 8 weeks post infection (2AII, 2AIII, NeoII and NeoIII). 197 genes were significantly altered by LMP2A, including 143 up regulated genes (A) and 54 down regulated genes (B). Among the genes changed in the presence of LMP2A, there was a clear difference in gene expression between cells 2 weeks post infection (AI) compared to 8 weeks post infection (AII and AIII).

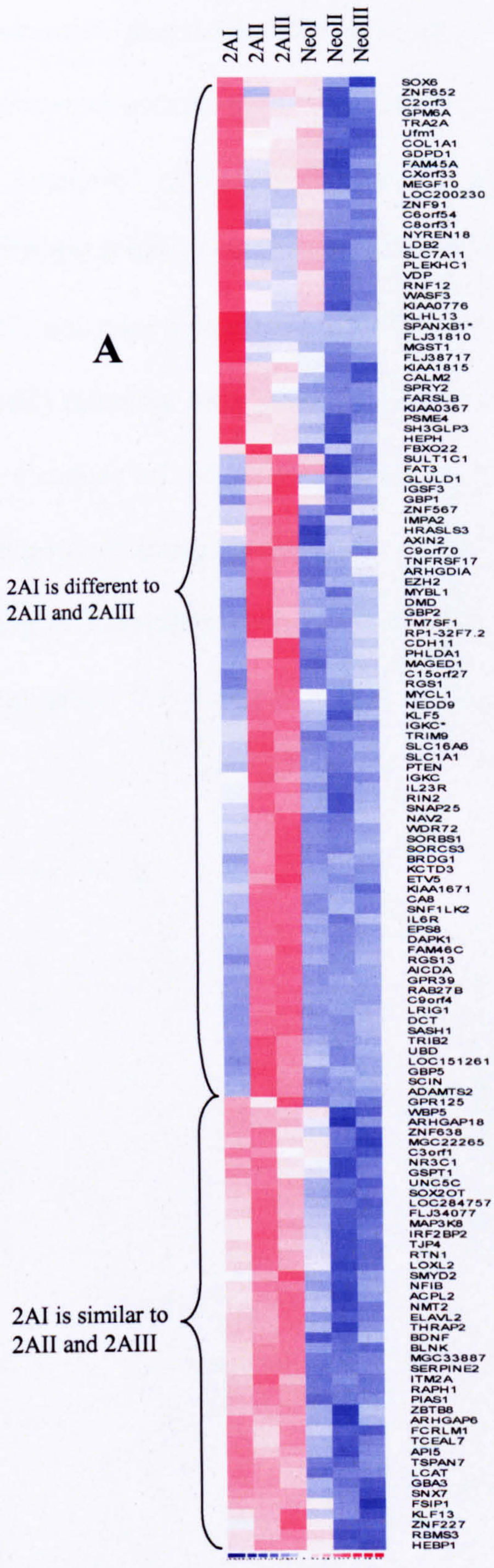

2AI
2AII
2AIII
NeoI
NeoII
NeoIII
A
2AI is different to
2AII and 2AIII
2AI is similar to
2AII and 2AIII
SOX6
ZNF652
C2orf3
GPM6A
TRA2A
Ufm1
COL1A1
GDPD1
FAM45A
CXorf33
MEGF10
LOC200230
ZNF91
C6orf54
C8orf31
NYREN18
LDB2
SLC7A11
PLEKHC1
VDP
RNF12
WASF3
KIAA0776
KLHL13
SPANXB1*
FLJ31810
MGST1
FLJ38717
KIAA1815
CALM2
SPRY2
FARSLB
KIAA0367
PSME4
SH3GLP3
HEPH
FBXO22
SULT1C1
FAT3
GLULD1
IGSF3
GBP1
ZNF567
IMPA2
HRASLS3
AXIN2
C9orf70
TNFRSF17
ARHGDIA
EZH2
MYBL1
DMD
GBP2
TM7SF1
RP1-32F7.2
CDH11
PHLDA1
MAGED1
C15orf27
RGS1
MYCL1
NEDD9
KLF5
IGKC*
TRIM9
SLC16A6
SLC1A1
PTEN
IGKC
IL23R
RIN2
SNAP25
NAV2
WDR72
SORBS1
SORCS3
BRDG1
KCTD3
ETV5
KIAA1671
CA8
SNF1LK2
IL6R
EPS8
DAPK1
FAM46C
RGS13
AICDA
GPR39
RAB27B
C9orf4
LRIG1
DCT
SASH1
TRIB2
UBD
LOC151261
GBP5
SCIN
ADAMTS2
GPR125
WBP5
ARHGAP18
ZNF638
MGC22265
C3orf1
NR3C1
GSPT1
UNC5C
SOX2OT
LOC284757
FLJ34077
MAP3K8
IRF2BP2
TJP4
RTN1
LOXL2
SMYD2
NFIB
ACPL2
NMT2
ELAVL2
THRAP2
BDNF
BLNK
MGC33887
SERPINE2
ITM2A
RAPH1
PIAS1
ZBTB8
ARHGAP6
FCRLM1
TCEAL7
API5
TSPAN7
LCAT
GBA3
SNX7
FSIP1
KLF13
ZNF227
RBMS3
HEBP1
B
2AI
2AII
2AIII
NeoI
NeoII
NeoIII
2AI is different to
2AII and 2AIII
2AI is similar to
2AII and 2AIII
MALAT1
CYP4X1
PTPRD
UNC13B
TMCC3
IFI27
PREX1
FAM9B
DPYS
SEPP1
SEMA6A
CST11
PRG4
TIAM1
SOX9
CPNE4
ESRRG
FABP4
ITIH5
FRMD4A
APP
RPRM
TPO
CHN2
FGF13
LOC553137
COL21A1
KIAA0992
ARG99
C1orf150
C6orf118
NFAT5
UHMK1
TCERG1
SFRS6
NAPSB
FLJ21986
SRGAP2
DSC1
BMP10
DKFZP434J0113
FLJ22457
ELK1
GAGE3
GAGE4*
GAGE5*
GAGE2*
GAGE1*
C21orf42
ID3
KIAA1202
FSTL5
MOSC2
PRL
JAM3
CYP4Z1
FLJ32096
LASS6

Figure 4-2 Heatmap of microarray data comparing cellular gene expression between LMP2B-expressing KMH2 cells and vector only control cells

Colour intensities represent the expression level of the three biological replicates; increasing intensity of red or blue represent increased or decreased expression relative to the mean (white) in LMP2B-expressing KMH2 cells compared to vector only control cells. Each experiment was performed in triplicate using RNA from separate flasks of cells, one flask of which were 2 weeks post infection (2BI and NeoI) and another two flasks of which were 8 weeks post infection (2BII, 2BIII, NeoII and NeoIII). 49 genes were significantly altered by the presence of LMP2B, including 47 genes up regulated and 2 down regulated genes. Among the genes changed in the presence of LMP2A, there was a clear difference in gene expression between cells 2 weeks post infection (BI) compared to 8 weeks post infection (BII and BIII).

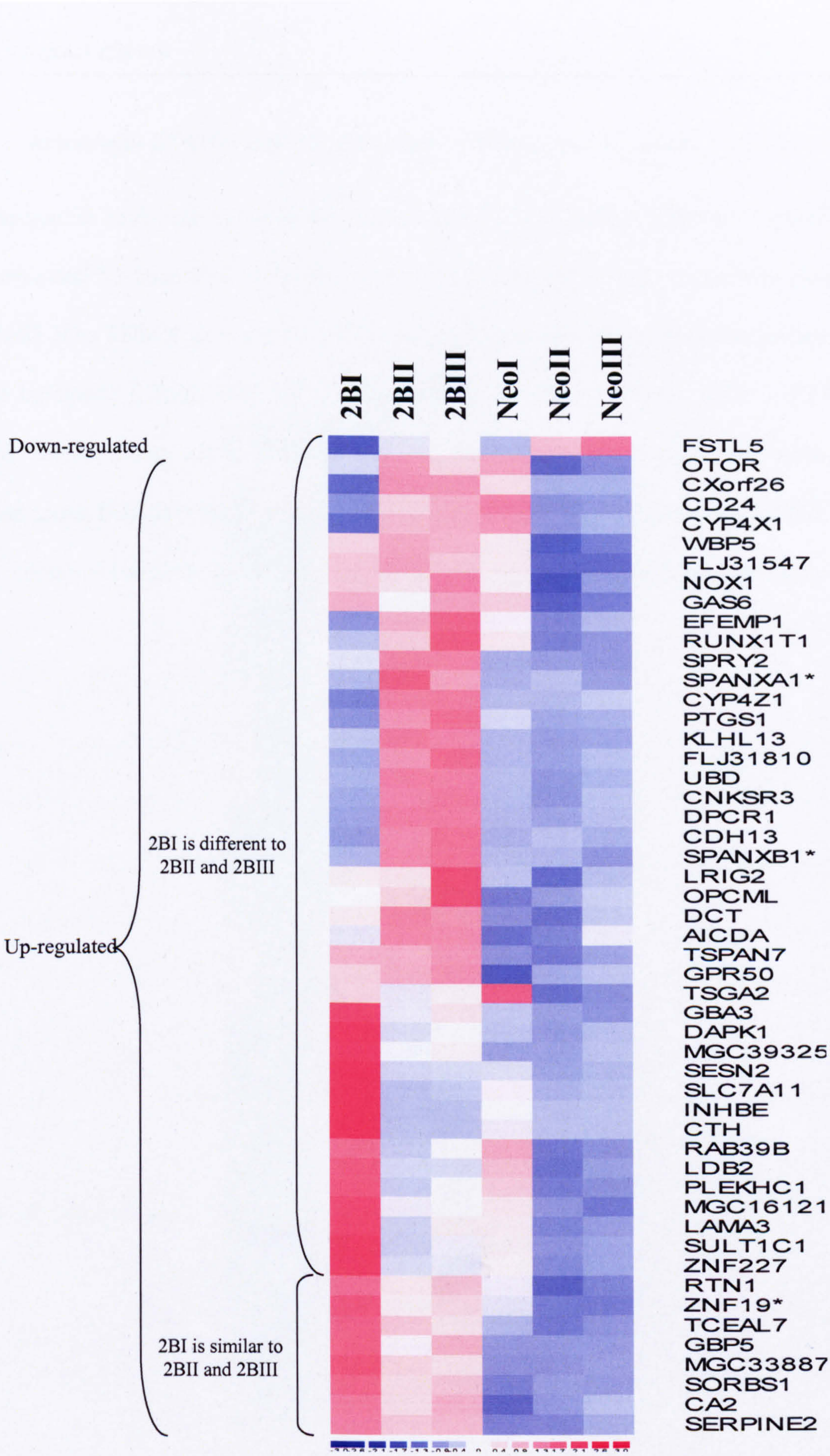

2BI
2BII
2BIII
NeoI
NeoII
NeoIII
Down-regulated
2BI is different to 2BII and 2BIII
Up-regulated
2BI is similar to 2BII and 2BIII
FSTL5
OTOR
CXorf26
CD24
CYP4X1
WBP5
FLJ31547
NOX1
GAS6
EFEMP1
RUNX1T1
SPRY2
SPANXA1*
CYP4Z1
PTGS1
KLHL13
FLJ31810
UBD
CNKSR3
DPCR1
CDH13
SPANXB1*
LRIG2
OPCML
DCT
AICDA
TSPAN7
GPR50
TSGA2
GBA3
DAPK1
MGC39325
SESN2
SLC7A11
INHBE
CTH
RAB39B
LDB2
PLEKHC1
MGC16121
LAMA3
SULT1C1
ZNF227
RTN1
ZNF19*
TCEAL7
GBP5
MGC33887
SORBS1
CA2
SERPINE2

Figure 4-3 Cellular genes altered by both LMP2A and LMP2B expression

Colour intensities represent the mean expression level of the three biological replicates; increasing intensity of red or blue represent increased or decreased expression relative to the mean (white) in LMP2A-expressing KMH2 cells (2AI, 2AII, 2AIII), LMP2B-expressing KMH2 cells (2BI, 2BII, 2BIII,) compared to vector only control cells (NeoI, NeoII, NeoIII). 49% of the genes altered by LMP2B were also changed by LMP2A. These included 23 up regulated genes and 1 down regulated gene. Gene symbol relative to Neo control of these are listed.


LMP2AI
LMP2AII
LMP2AIII
LMP2BI
LMP2BII
LMP2BIII
NeoI
NeoII
NeoIII
FSTL5
SPRY2
SPANXB
KLHL13
FLJ31810
SLC7A11
LDB2
PLEKHC1
UBD
DCT
AICDA
WBP5
SULT1C1
GBA3
TSPAN7
TCEAL7
MGC33887
SERPINE2
SORBS1
DAPK1
GBP5
PTN1
ZNF227

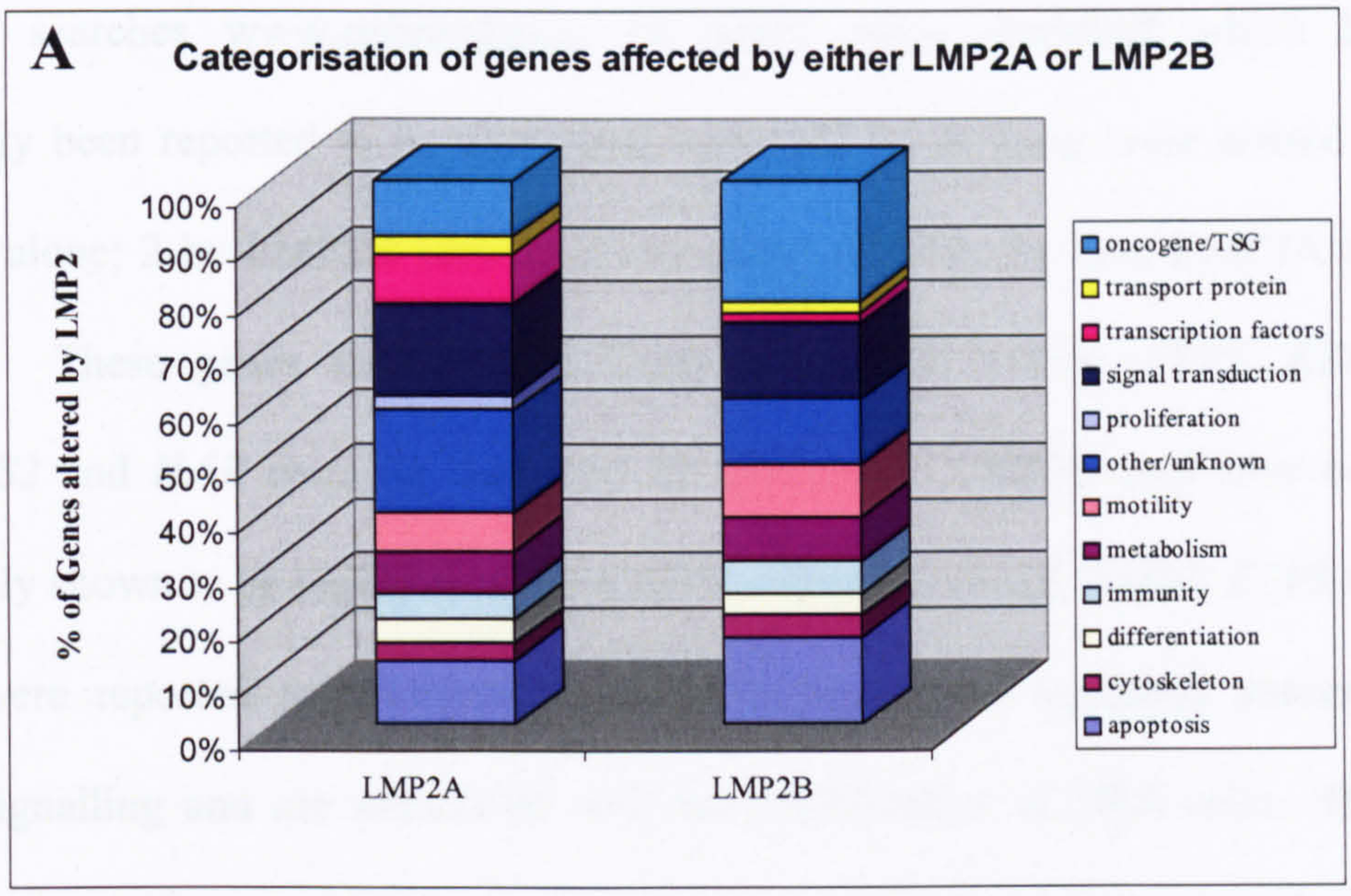
4.2.2 Classification of genes regulated by LMP2

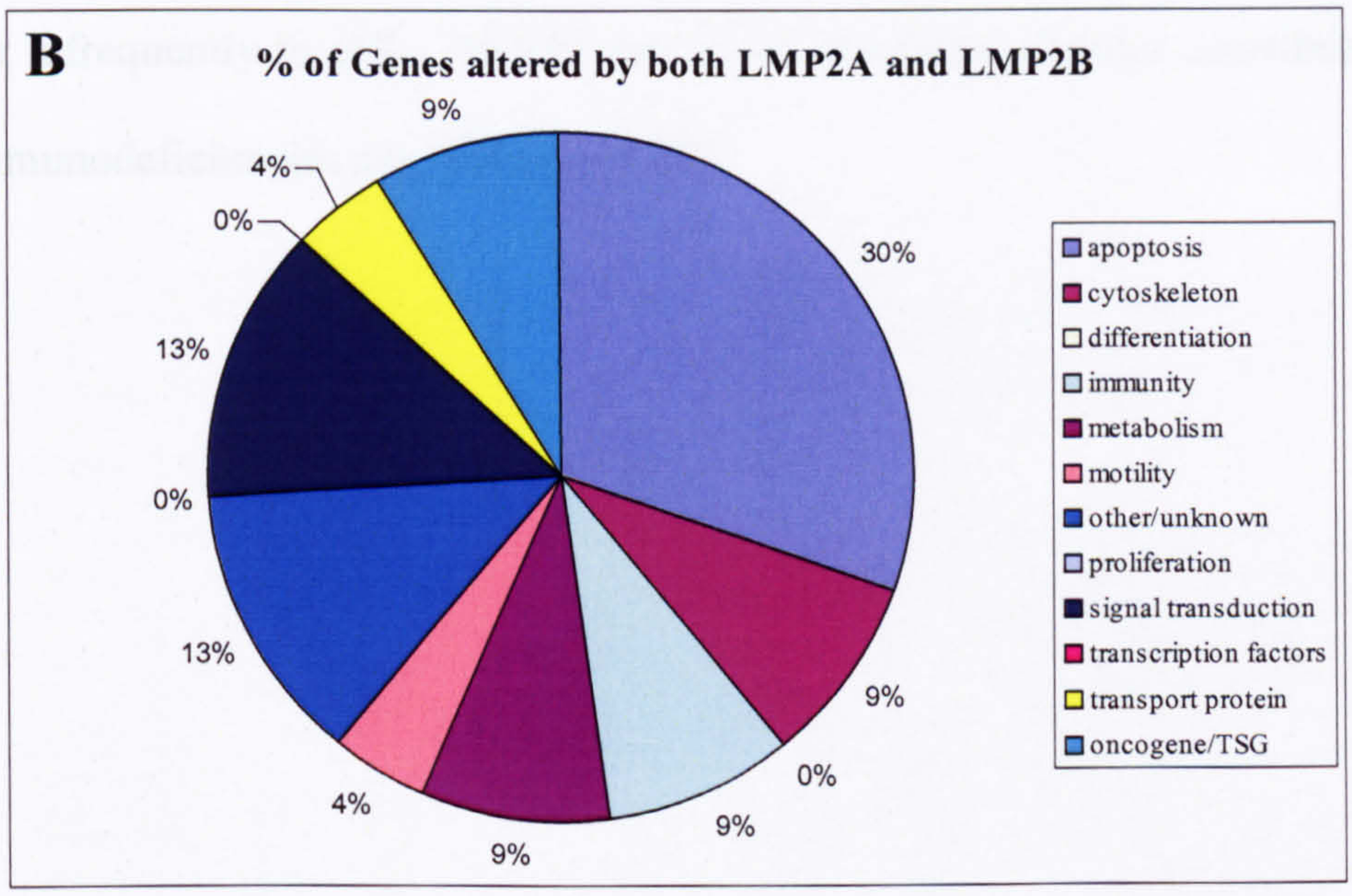
In order to further define the effects of LMP2 in HL cells, genes identified from each microarray were placed into the following functional categories; anti-apoptosis, cytoskeleton, metabolism, immunity, signal transduction, transcription, transport protein and oncogenes/TSG (tumour suppressor gene). Those genes whose function was unknown or that could not be classified into one of the above categories were designated other/unknown. Gene function was ascertained from published papers at www.pubmed.gov. The lists of genes changed by LMP2A or LMP2B are presented in the Appendix (Table 8.1 and Table 8.2). A summary of gene function influenced by LMP2A, LMP2B or both, is given in Figure 4.4 A and Figure 4.4 B. Overall, the proportions of genes categorised as having a function associated with cytoskeleton, differentiation, immunity, metabolism, and transport protein were very similar, but the contributory genes were often different. A larger proportion of genes involved in signal transduction and transcription were altered by LMP2A compared with LMP2B. In contrast, a higher proportion of genes involved in motility, apoptosis and categorised oncogenes/TSG, were altered by LMP2B expression compared with LMP2A. Figure 4.4 B shows that 30% of the genes that were differentially expressed in both LMP2A and LMP2B cells might suggest a role for LMP2A and LMP2B in the rescue of pre-apoptotic germinal centre B cells.

Figure 4-4 Sub-categorisation of genes altered by LMP2A and LMP2B

(A) Shows the relative proportion of genes within each functional category influenced by either LMP2A or LMP2B.

(B) Shows that the relative proportion of genes within each functional category influenced by both LMP2A and LMP2B.


A
Categorisation of genes affected by either LMP2A or LMP2B
% of Genes altered by LMP2
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
LMP2A
LMP2B
oncogene/TSG
transport protein
transcription factors
signal transduction
proliferation
other/unknown
motility
metabolism
immunity
differentiation
cytoskeleton
apoptosis


B
% of Genes altered by both LMP2A and LMP2B
9%
4%
0%
13%
0%
13%
4%
9%
9%
0%
9%
30%
9%
apoptosis
cytoskeleton
differentiation
immunity
metabolism
motility
other/unknown
proliferation
signal transduction
transcription factors
transport protein
oncogene/TSG

4.2.3 Correlation of differentially expressed genes with those reported to be associated with Hodgkin's lymphoma

Cellular genes differentially expressed in the presence of LMP2A or LMP2B were annotated on the basis of any previously reported association with HL using PubMed searches www.pubmed.gov. 14 genes were identified which had previously been reported to be associated with HL; 11 of these were altered by LMP2A alone; 2 by LMP2B alone and one gene, AICDA, by both LMP2A and LMP2B. These genes are listed in Table 4.1. *GAS6, CD24, ETV5, EZH2, ADAMTS2* and *IL6R* were up regulated by LMP2A or LMP2B and have been previously shown to be highly expressed in HL, of which *GAS6, CD24, ETV5* and *EZH2* were reported as oncogenes. *ADAMTS2* and *IL6R* regulated abnormal STAT signalling and are associated with the proliferation of HRS cells. Both *BLNK* and *AICDA* are highly expressed in lymphocyte-predominant HL, but absent or infrequently in cHL. *HEBP1* acts as an immunomodulator contributing to the immunodeficiencies associated with HL.

Table 4-1 Cellular genes changed by LMP2A or LMP2B and also previously reported to be associated with HL

Gene Symbol	Gene Title	Fold	LMP2	Reference	Summary
PRL	prolactin	-2.3	LMP2A	PMID: 9458932 PMID: 2038709	Prolactin was used as hormane indicator of reproductive and ovarian function of Hodgkin's lymphoma patients with chemotherapy and radiotherapy
TPO	thyroid peroxidase	-1.9	LMP2A	PMID: 9226215	Hodgkin's lymphoma patients display altered TPO antibody-dependent immune function with chemotherapy and radiotherapy
ELK1	ELK1, member of ETS oncogene family	-1.8	LMP2A	PMID: 6226085	ELK1 related with a lower number of T lymphocytes was demonstrated in HL patients with advanced disease
LCAT	lecithin-cholesterol acyltransferase	1.5	LMP2A	PMID: 3768760	Synthesis or release of lecithin:cholesterol acyltransferase was found in a HL patient after chemotherapy
ETV5	ets variant gene 5 (ets-related molecule)	1.8	LMP2A	PMID: 15632006	ERM expression was observed in all HL and LBCL cell lines and related with the production of various cytokines
IL6R	interleukin 6 receptor	2.2	LMP2A	PMID: 11468177 PMID: 10379062	IR6R is expressed in HL cell lines, but antibody against IL-6R did not inhibit the DNA-binding activity of STAT3
HEBP1	heme binding protein 1	1.8	LMP2A	PMID:2690234 PMID: 3567932	HBP recognize the Hodgkin's lectin, which suggests its role as an immunomodulator contributing to the immunodeficiencies associated with Hodgkin's disease.
BLNK	B-cell linker	1.9	LMP2A	PMID: 12881301 PMID: 15744341	BLNK was consistently absent from Reed-Sternberg cells in classical Hodgkin's lymphoma, but it was positive in the lymphocyte predominance Hodgkin's lymphoma.
EZH2	Enhancer of zeste homolog 2 (Drosophila)	1.9	LMP2A	PMID:14982841 PMID: 10980109	Co-expression of BMI-1 and EZH2 in Hodgkin/Reed-Sternberg (H/RS) cells contributes to development of HL
DMD	dystrophin (muscular dystrophy, Duchenne and Becker types)	1.9	LMP2A	PMID: 14707720	A Hodgkin's lymphoma patient was associated with Becker muscular dystrophy
ADAMTS2	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 2	2.2	LMP2A	PMID: 11468177 PMID: 10379062	Expressed in Hodgkin's lymphoma cell lines and regulated abnormal STAT signalling and growth
AICDA	activation-induced cytidine deaminase	6.8	LMP2A/2B	PMID:16126891 PMID: 15732141	Aid related with somatic hypermutation may remain active in L&H cells, increasing the risk of acquiring further transforming mutations. AID is consistently expressed in the neoplastic cells of nodular lymphocyte-predominant Hodgkin lymphoma (HLnlp) but only infrequently in classical HL (cHL).
GAS6	Growth arrest-specific 6	1.7	LMP2B	PMID: 10400186	GAS6 was expressed in a proportion of T-, B- original lymphoma cell lines and Hodgkin's lymphoma cell lines suggested GAS6 caused disordered growth or chemotaxis/adhesion of leukemia and lymphomas.
CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	1.3	LMP2B	PMID: 9935206 PMID: 1531569	CD24 expressed in the Hodgkin's lymphma and LCL cells.

4.2.4 Cellular genes altered by LMP2A or LMP2B previously reported to be associated with EBV

Differentially expressed genes were also annotated according to any previous reports of their association with EBV. 12 genes had been reported in association with EBV; 8 of which were altered by LMP2A and 4 by LMP2B. These are listed in Table 4.2. Four genes, *ID3, IL6R, GAS6* and *CD24* were also reported to be associated with HL (section 4.2.3). *BDNF* and *NR3C1* were both up regulated by LMP2A and have been detected in EBV transformed B cells. *MAP3K8 (Tpl2)* is a component of LMP1 signalling downstream of TRAF2 and was upregulated by LMP2A. ID3 is downregulated by LMP2A but upregulated by LMP1. Three genes (PTGS1, *GAS6* and *CD24*) were up regulated by LMP2B were all oncogenes detected in EBV transformed cell lines.

Table 4-2 Cellular genes targeted by LMP2 also previously reported to be associated with EBV

Gene Symbol	Gene Title	Fold change	LMP2	Reference	Summary
GAGE2	G antigen 2	-2.4	LMP2A	PMID: 10928100	EBV infection appears to induce IFN-gamma gene expression in most of undifferentiated carcinoma of nasopharyngeal type (UCNT), but GAGE-1/-2 expression in only some tumours.
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	-1.7	LMP2A	PMID: 15564458	LMP1 transduced human cervical carcinoma cell line C33A induced high expression of ID1 and ID3.
PIAS1	Protein inhibitor of activated STAT, 1	1.5	LMP2A	PMID: 15229220	Epstein-Barr virus (EBV) expresses an immediate-early protein, Rta, to activate the transcription of EBV lytic genes and the lytic cycle. Ubc9 and PIAS1 were identified as binding partners of Rta.PIAS1 stimulates conjugation of SUMO-1 to Rta and indicates that sumoylation of Rta may be important in EBV lytic activation.
COL1A1	collagen, type I, alpha 1	1.6	LMP2A	PMID: 10942108	Osteogenesis imperfecta (OI) is an autosomal dominant genetic disorder as a result of mutations in the genes that encode the chains of type I collagen, the major protein of bone. EBV transformed lymphoblasts resulted in 87% and 85% for OI type I and OI type IV respectively and the allele drop-out (ADO) rate was assessed at 11.5% and 11.1% for OI type I and OI type IV respectively.
BDNF	brain-derived neurotrophic factor	1.7	LMP2A	PMID: 8632056	EBV transformed B cells express detectable levels of transmembrane tyrosine kinase b(Trk b) and its mRNA. Exposure of EBV-transformed B lymphocytes to brain-derived neurotrophic factor (BDNF) triggered the phosphorylation of Trk b.
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	1.7	LMP2A	PMID: 11932422	Tpl-2 as a component of LMP1 signaling downstream of TRAF2 and as a modulator of LMP1-mediated effects.
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	1.8	LMP2A	PMID: 3002935 PMID: 2157866	EBV transformation is associated with increases in the concentration and the absolute number of glucocorticoid receptors
IL6R	interleukin 6 receptor	2.2	LMP2A	PMID: 11468177 PMID: 10379062	IL6R is expressed in HL cell lines, but antibody against IL-6R did not inhibit the DNA-binding activity of STAT3
CDH13	cadherin 13, H-cadherin (heart)	2.5	LMP2B	PMID: 15172980	Promoter methylation of CDH1 and CDH13 were noted in dual SV40- and EBV-infected PBMC, and these two genes were also highly significantly correlated to the presence of SV40 sequences in tumors.
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	2.1	LMP2B	PMID: 10843703	EBV was found to impact on the NF-kappaB activation pathway, which plays an essential role in the induction of COX-2 in monocytes.
GAS6	Growth arrest-specific 6	1.7	LMP2B	PMID: 10400186	GAS6 was expressed in a proportion of T-, B-original lymphoma cell lines and Hodgkin's lymphoma cell lines suggested GAS6 caused disordered growth or chemotaxis/adhesion of leukemia and lymphomas.
CD24	CD24 antigen (small cell lung carcinoma cluster4 antigen)	1.3	LMP2B	PMID: 9935206 PMID: 1531569	CD24 expressed in the Hodgkin's lymphma and LCL cells.

4.2.5 LMP2A up regulates some B cell markers lost in HL cell lines

17 genes up regulated in LMP2A expressing KMH2 cells were previously shown to be down regulated in HL. 9 of these genes were involved in B cell receptor signalling. 2 genes (*ID3, NAPSB*) were down regulated and 3 genes (*COL1A1, IGSF3, SLC7A11*) were up regulated in both LMP2A expressing cells and HL cells (Table 4.3).

BLNK and *BRDG1* are docking proteins that function in downstream BCR signalling. *BLNK* expression was reported to be lost in HRS cells (Schwering *et al.*, 2003). *IGKC/V* and *AICDA* are involved in gene rearrangement; *AICDA*, initiates somatic hypermutation in B cells. *NEDD9* and *RGS3* regulate B cell adhesion, migration and the immune response. *PTPRC*, *MYBL1* and *TNFRSF17* function as transcriptional transactivators and enhance proliferation. Taken together, these data suggest that LMP2A might upregulate specific components of BCR signalling to provide survival and anti-apoptotic signals to allow the survival of HRS cells in the absence of BCR expression.

Table 4-3 LMP2A regulated genes known to be upregulated or downregulated in HL

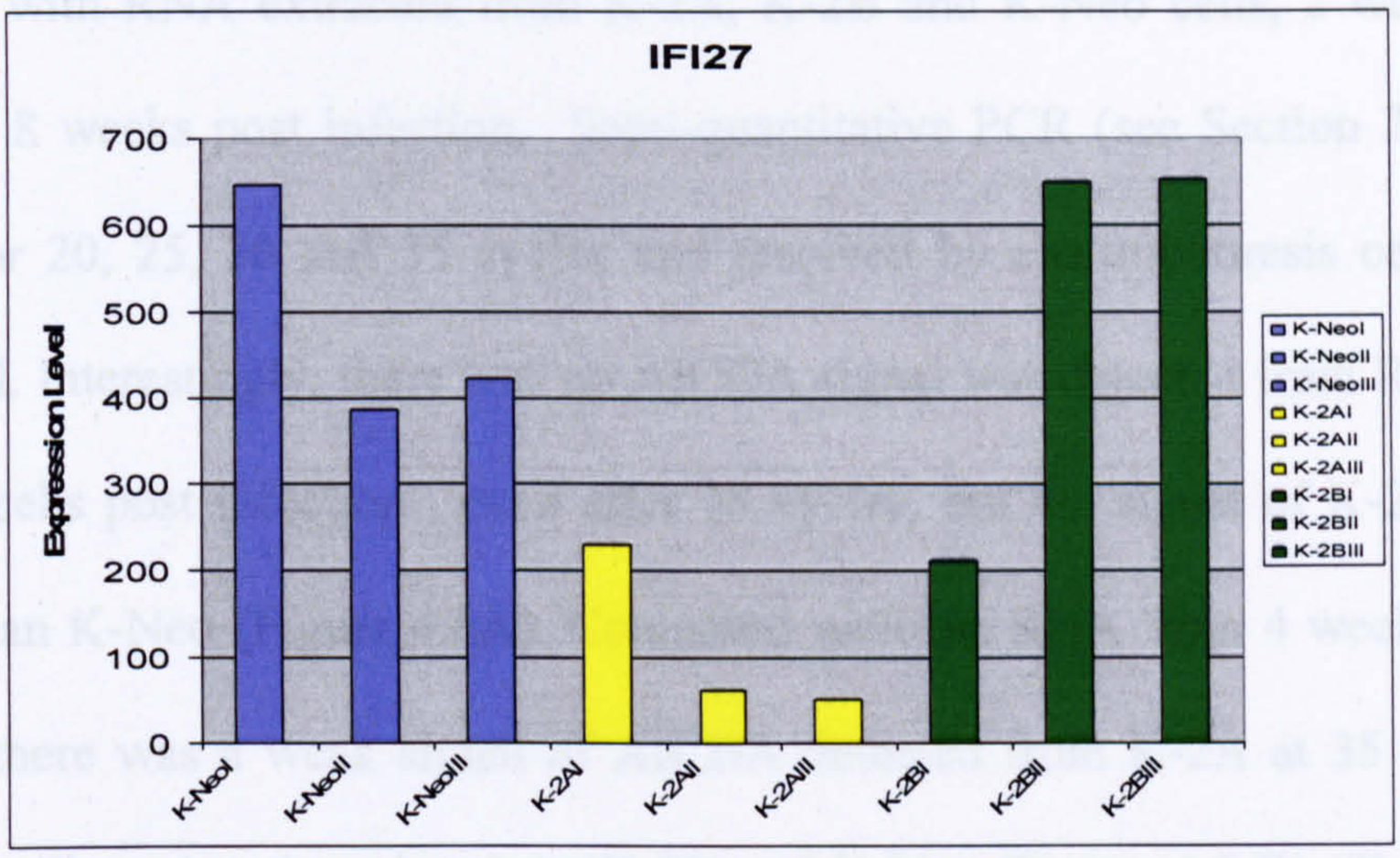
Gene	Gene title	BCR	Fold	Function	Reference
Down in HL, up in LMP2A					
PTPRC	protein tyrosine phosphatase, receptor type, C	YES	1.6	CD45 exiting widely in T and B cells has an important role in the early events of receptor signalling	PMID:15622930 PMID:12588355
MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	YES	2.1	Functioning as transcriptional transactivators and enhancing peripheral B cell survival or proliferative capacity	PMID:10910917 PMID:11207276
TNFRSF17	tumor necrosis factor receptor superfamily, member 17	YES	2.1	B cell maturation Ag (BCMA), a member of the TNFR superfamily expressed on B cells, binds to a proliferation-inducing ligand (APRIL) and activates the JNK pathway.	PMID:16116167 PMID:14978135 PMID:15070697
IGKC/V5	immunoglobulin kappa constant ///variable 1-5	YES	2.4	Gene rearrangement	PMID:1961033
IGHG1	Immunoglobulin heavy constant gamma 1	YES	1.5	Immunity	PMID:9531163
BLNK	B-cell linker	YES	1.9	BLNK is a component of BCR as well as of the pre-BCR signalling pathway.	PMID:12881301 PMID:15744341
BRDG1	BCR downstream signaling 1	YES	2.2	BRDG1 functions as docking protein acting downstream of Tec family (PTKs) in BCR signalling.	PMID:10518561 PMID:12540842
AICDA	activation-induced cytidine deaminase	YES	6.8	AICDA initiates switch recombination and somatic hypermutation in B cells by targeted deamination of transcribed genes and contribute to the genetic instability of c-MYC in B-cell malignancies	PMID:15940261 PMID:15732141
NEDD9	neural precursor cell expressed, developmentally down-regulated 9	YES	1.8	HEF1-associated AND-34 regulates B cell adhesion and motility through a Cdc42-mediated signaling pathway	PMID:9498705 PMID:12517963
RGS13	regulator of G-protein signalling 13	YES?	1.8	RGS molecules in helping regulate cell positioning in lymphoid organs during the immune response, lymphocyte migration and chemokine receptor signalling.	PMID:15047827 PMID:15313557
NR3C1	Nuclear receptor subfamily 3,group C, member 1 (glucocorticoid receptor)	NO	1.8	Glucocorticoid receptor is a transcriptional regulator of cell death during tumorigenesis of fibrosarcoma development	PMID:12637494 PMID:15770078
TRA2A	Transformer-2 alpha	NO	1.6	differentiation	PMID:15210204
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1	NO	2.5	LRIG1 is a negative regulator of ErbB family receptor tyrosine kinases and links to c-Cbl and receptor ubiquitylation	PMID:15686631 PMID:15345710
ITM2A	integral membrane protein 2A	NO	2.3	A marker for early stages in chondrogenesis and T-cell development. ITM2A enhances myogenic differentiation in vitro.	PMID:15325075 PMID:14984746
KLF13	Kruppel-like factor 13	NO	1.6	KLF13's repression of LDLR promoter activity regulates Cholesterol homeostasis	PMID:16303770 PMID:15716005
ZNF91	zinc finger protein 91 (HPF7, HTF10)	NO	1.6	ZFP91 is likely to play an important role in cell proliferation and/or anti-apoptosis in acute myelogenous leukemia	PMID:12738986 PMID:11470777
FAM46C	family with sequence similarity 46, member C	NO	2.1		
Down in HL, down in LMP2A					
ID3	inhibitor of DNA binding 3	YES	-1.7	ID3 blocks B lymphocyte maturation at an early differentiation step and works as an antaganist of E protein	PMID:6297338 PMID:15769946
NAPSB	napsin B aspartic peptidase pseudogene	NO	-1.7	metabolism	PMID:16162398 PMID:14674886
Up in HL, up in LMP2A					
COL1A1	collagen, type I, alpha 1	NO	1.6	Osteogenesis imperfecta is secondary to mutations of COL1A1 and COL1A2.	PMID:15339338 PMID:11479215
IGSF3	immunoglobulin superfamily, member 3	NO	1.7	IGSF3 contributes to a variety of CD9 and CD81 functions in virus-induced cell-cell fusion and virus entry into and/or release.	PMID:11504738
SLC7A11	solute carrier family 7, member 11	NO	1.7	Mediates cystine-glutamate exchange and regulates intracellular glutathione levels.	PMID:16103098

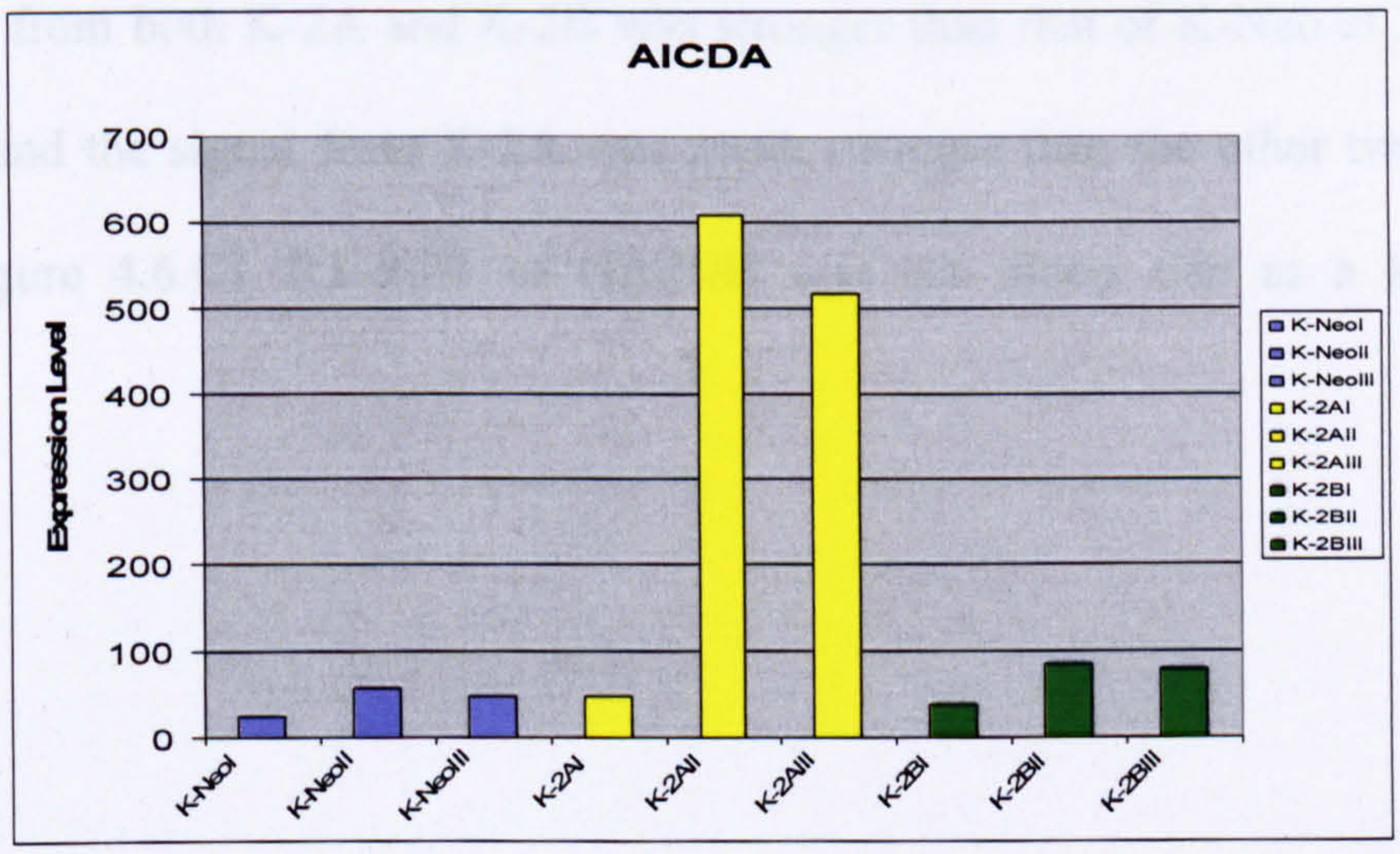
4.2.6 Expression of IFI27 and AICDA as determined by GCOS signal from microarrays

Two genes were selected for initial validation, IFI27 and AICDA. GCOS signals for each gene were plotted and are shown in Figure 4.5. This result shows that although AICDA was up regulated in both LMP2A and LMP2B-expressing KMH2 cells, the upregulation is only convincing in the later passages of LMP2A-expressing cells. The expression of IFI27 was down regulated by LMP2A, but not LMP2B.

Figure 4-5 GCOS signals of IFI27 and AICDA detected on microarray

GCOS signals for each gene were plotted. This shows that although AICDA was up regulated in both LMP2A and LMP2B-expressing KMH2 cells, the upregulation is only convincing in the later passages of LMP2A-expressing cells. The expression of IFI27 was down regulated by LMP2A, but not LMP2B.


IFI27
Expression Level
700
600
500
400
300
200
100
0
K-Neol
K-NeolI
K-NeolII
K-2AI
K-2AII
K-2AIII
K-2BI
K-2BII
K-2BIII


AICDA
Expression Level
700
600
500
400
300
200
100
0
K-Neol
K-NeolI
K-NeolII
K-2AI
K-2AII
K-2AIII
K-2BI
K-2BII
K-2BIII

4.2.7 Validation of cellular gene targets of LMP2

4.2.7.1 Expression of AICDA detected by semi-Q-PCR in different passages of LMP2 positive KMH2 cells

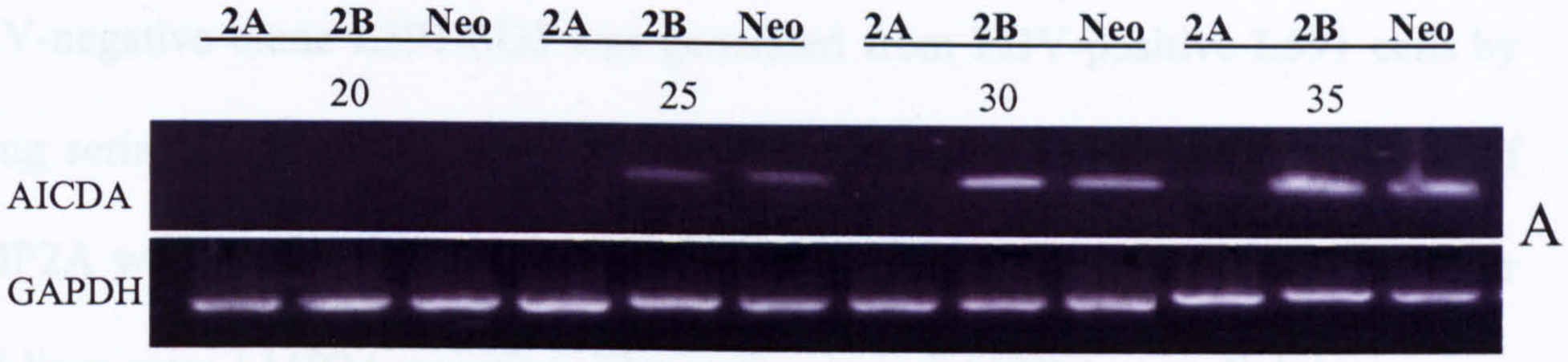
From the array result, AICDA was upregulated by LMP2A, so semi-Q-PCR was performed with RNA extracted from K-2A, K-2B and K-Neo cells, 2 weeks, 4 weeks and 8 weeks post infection. Semi-quantitative PCR (see Section 2.3.1.4) was run for 20, 25, 30 and 35 cycles and resolved by electrophoresis on 1.2% agarose gel. Interestingly, there was no AICDA signal was detected from RNA of K-2A 2 weeks post infection , even after 35 cycles, but the signal of K-2B was stronger than K-Neo (Figure 4.6A). Compared with the RNA from 4 weeks post infection, there was a weak signal of AICDA detected from K-2A at 35 cycles, but it was still weaker than that from K-2B and K-Neo (Figure 4.6 B). The same semi-Q-PCR was performed with the RNA from 8 weeks post infection, the signal of AICDA from both K-2A and K-2B was stronger than that of K-Neo at 30 and 35 cycles and the signal from K-2A was much stronger than the other two by 8 weeks (Figure 4.6.C). RT-PCR of GAPDH was run along side as a loading control.

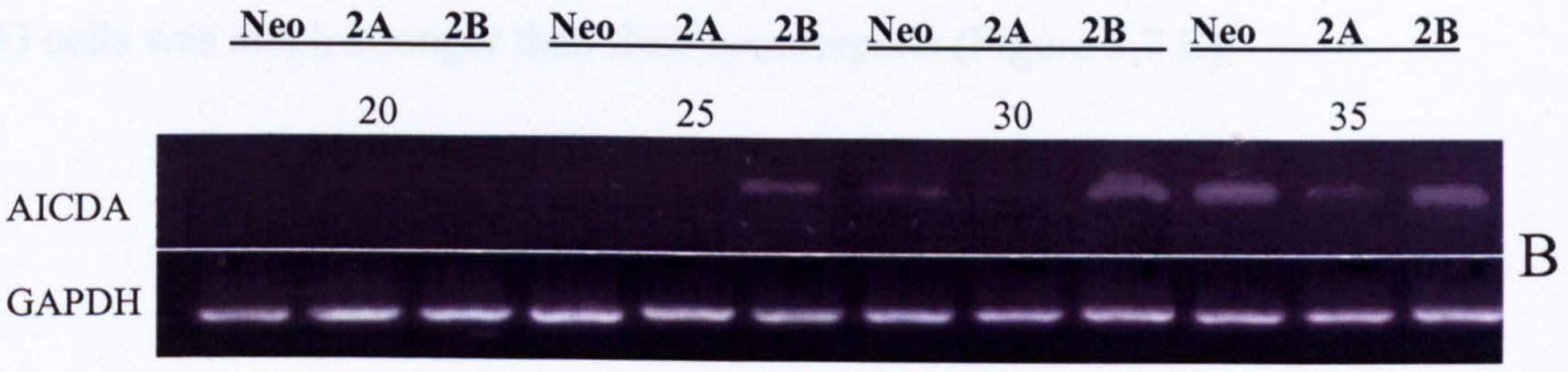
Figure 4-6 Expression of AICDA detected by semi-Q-PCR

Semi-Q-PCR was performed with RNA extracted from K-2A, K-2B and K-Neo cells at 2 weeks, 4 weeks and 8 weeks post infection for 20, 25, 30 and 35 cycles. (A) AICDA expression was not detectable in LMP2A-expressing cells at 2 weeks post infection.

(B) At 4 weeks AICDA expression was still weaker in K-2A cells than in K-2B and K-Neo cells.

(C) However, at 8 weeks AICDA expression was stronger in both K-2A and K-2B in K-Neo cells.


2A 2B Neo 2A 2B Neo 2A 2B Neo 2A 2B Neo
20 25 30 35
AICDA
GAPDH
A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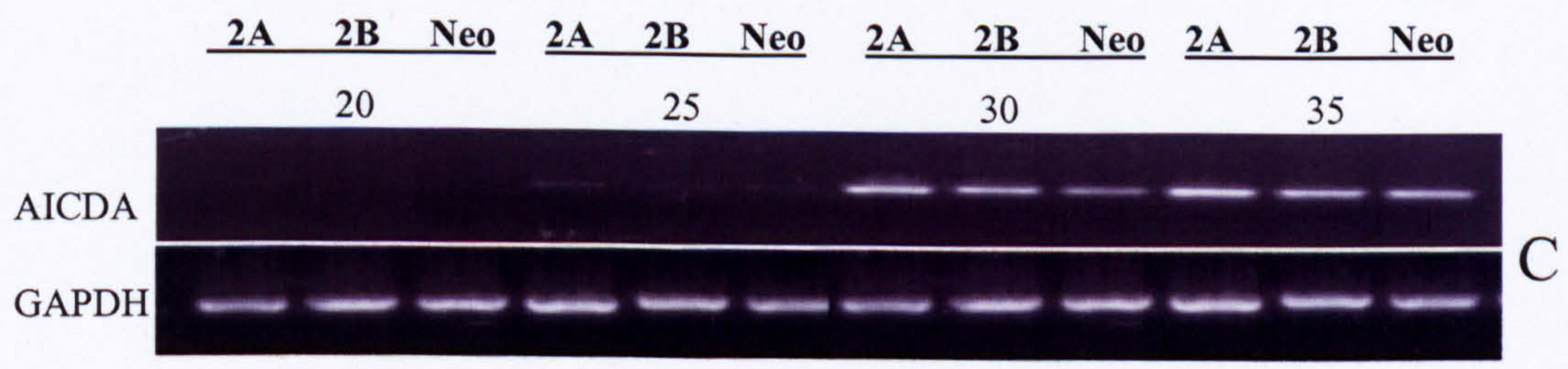
20 25 30 35
AICDA
GAPDH
B


2A 2B Neo 2A 2B Neo 2A 2B Neo 2A 2B Neo
20 25 30 35
AICDA
GAPDH
C

4.2.7.2 Expression of AICDA in HL cell lines

As described in Chapter 3, the expression level of LMP2A in K-2A cells was lower in the early passages. The expression of LMP2A in K-2A cells 8 weeks post infection was compared with EBV positive HL cell lines KMH2-EBV (K-EBV), L591 and their EBV-negative counterparts KMH2 and L-591-SD3 (Figure 4.7 A). EBV-negative clone L591-SD3 was generated from EBV-positive L591 cells by being serially diluted for up to 6 weeks (Baumforth *et al,* 2005). Expression of LMP2A was strong in K-2A cells and weak expression in L591 cells. The other cell lines were LMP2A-negative. The expression of AICDA was slightly stronger in KMH2 cells than in K-EBV cells (Figure 5.7 B). The same assay was performed with L591 and L591-SD3 cells. The expression of AICDA in L591-SD3 cells was much stronger than their counterparts (Figure 5.7 C).

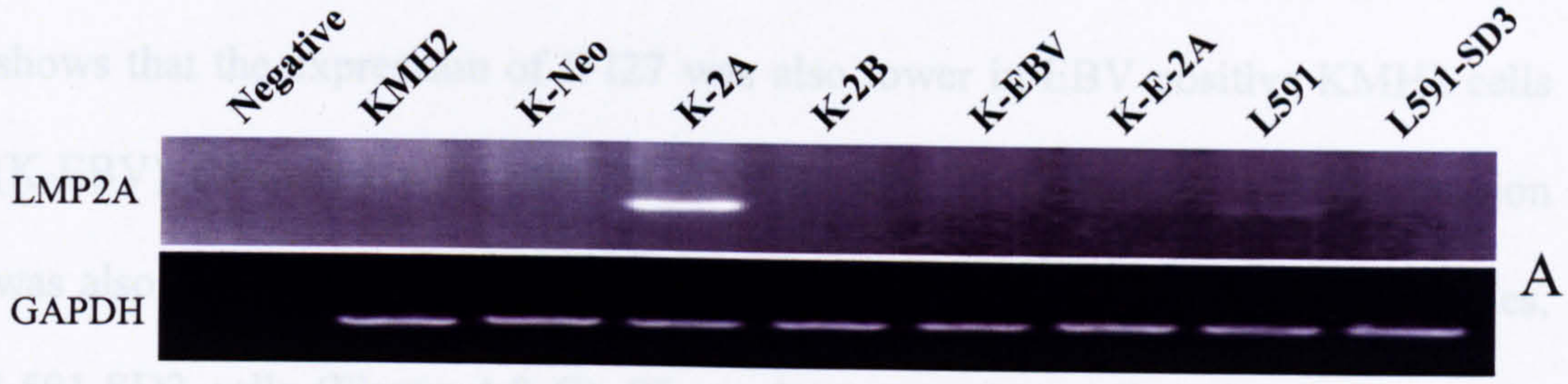
Figure 4-7 Expression level of LMP2A and AICDA detected by RT-PCR

The expression of LMP2A was compared by RT-PCR in K-2A cells 8 weeks post infection and in a panel of with other EBV positive HL cell lines compared with their EBV-negative counterparts.

(A) K-2A cells showed strong expression of LMP2A and a very weak one was detected in L591 cells.

(B) The expression of AICDA was slightly stronger in KMH2 cells than in K-EBV cells.

(C) The expression of AICDA in L591-SD3 was much stronger than in L-591.


Negative
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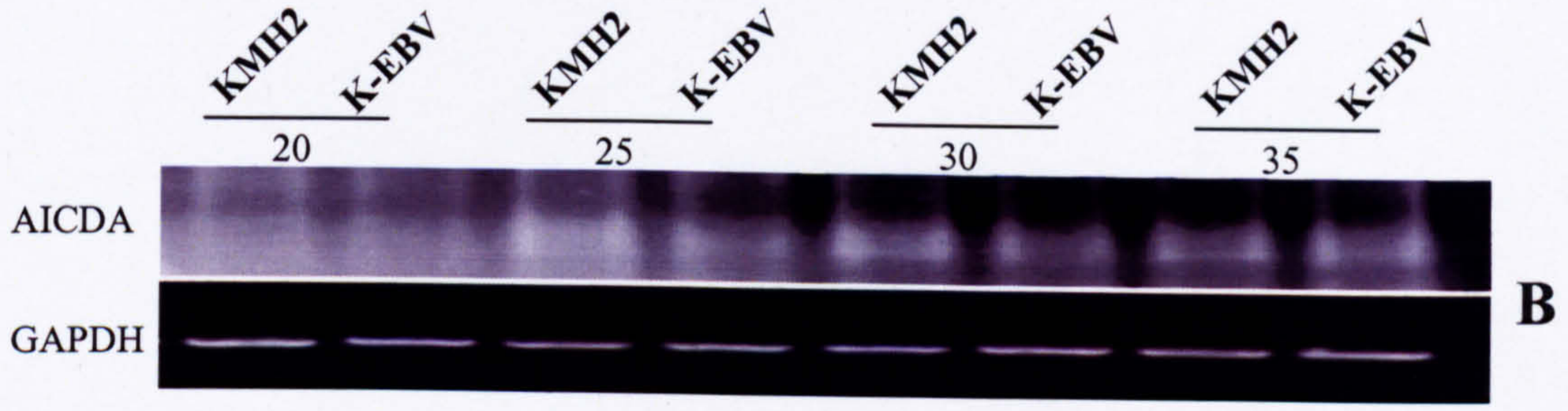
K-Neo
K-2A
K-2B
K-EBV
K-E-2A
L591
L591-SD3
LMP2A
GAPDH
A


KMH2
K-EBV
20
KMH2
K-EBV
25
KMH2
K-EBV
30
KMH2
K-EBV
35
AICDA
GAPDH
B


L591-SD3
L591
20
L591-SD3
L591
25
L591-SD3
L591
30
L591-SD3
L591
35
AICDA
GAPDH
C

4.2.7.3 Expression of IFI27 in HL cell lines

By array analysis, IFI27 expression was down regulated in K-2A cells, but not in K-2B cells. Figure 4.8 A shows that IFI27 mRNA levels at two weeks post infection were lower in K-2A than in either K-2B or K-Neo. The expression of IFI27 in K-2A was not different at 2 and 8 weeks (data not shown). Figure 4.8 B shows that the expression of IFI27 was also lower in EBV-positive KMH2 cells (K-EBV) compared with parental KMH2 cells. Furthermore, IFI27 expression was also lower in EBV-positive L591 cells compared with EBV-negative clones, L591-SD3 cells (Figure 4.8 C). These data not only confirm the results of the array analysis, but also suggest that LMP2A also downregulates IFI27 in the presence of EBV infection.

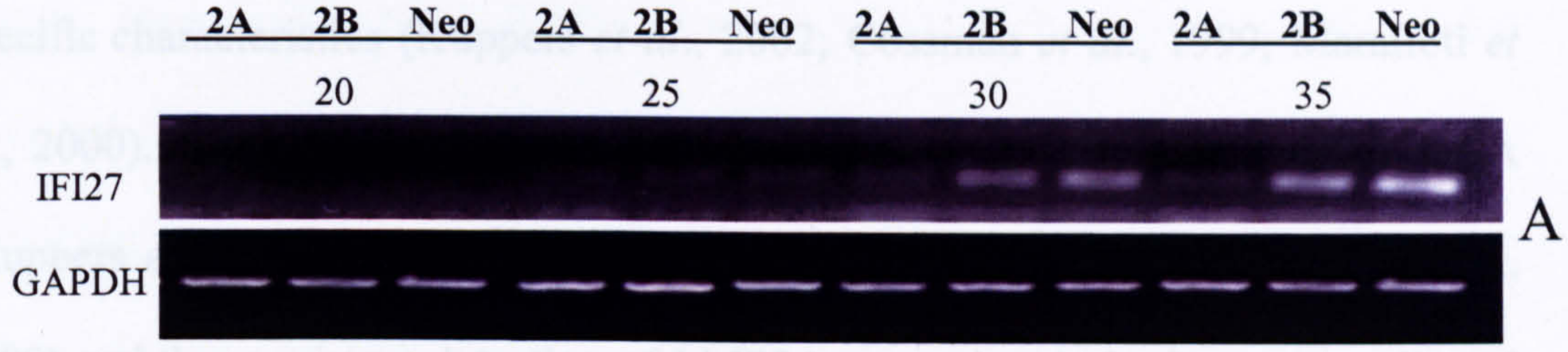
Figure 4-8 Expression of IFI27 in HL cell lines

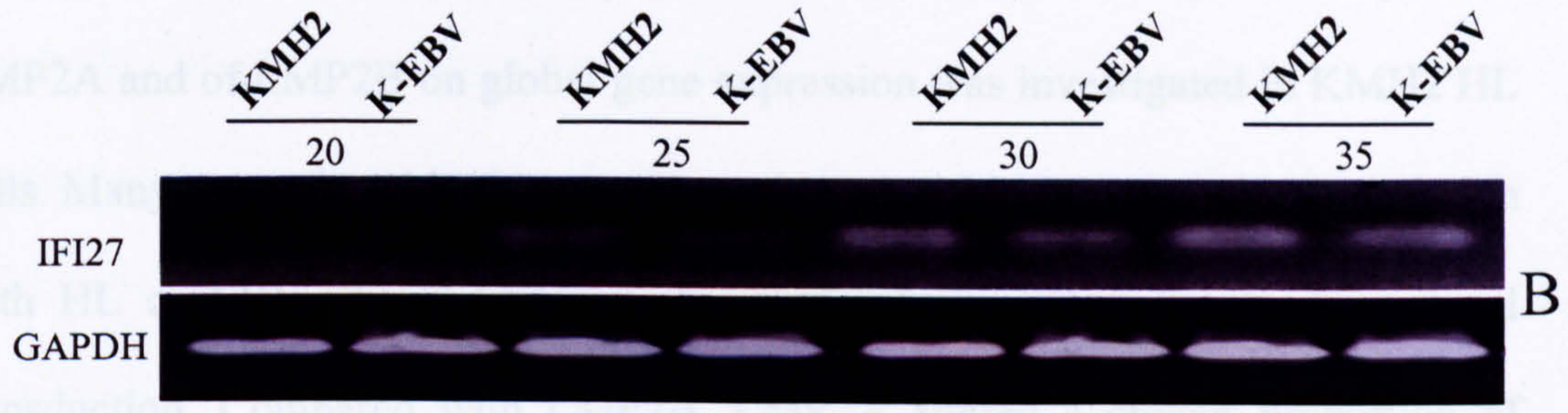
IFI27 expression was studied by semi-Q-PCR. K-2A, K-2B and K-Neo two weeks and eight weeks post infection; recombinant EBV and its empty vector Neo infected KMH2 cells; EBV positive HL cell line L591 and its negative counterpart L591-SD3. RNA was extracted from all these cells and cDNA was made. Each cDNA sample was run semi-quantitative PCR for 20, 25, 30 and 35 cycles.

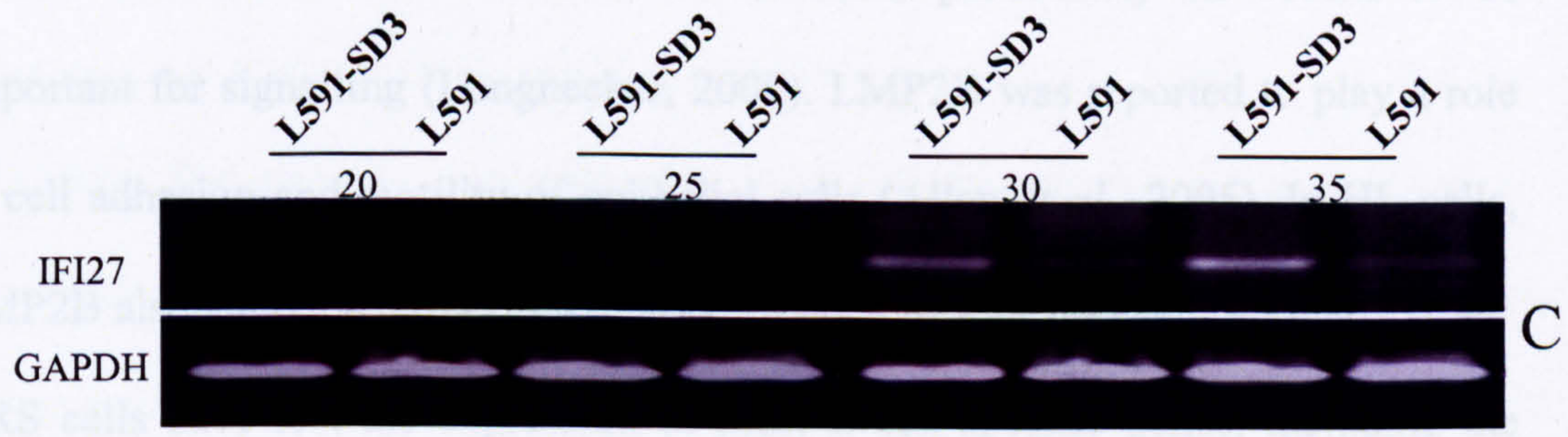
(A) The signal of K-2A was weaker than K-2B and K-Neo at 30 and 35 cycles.

(B) The signal of K-EBV was weaker than K-Neo at 25, 30 and 35 cycles.

(C) The signal of L591 was weaker than L591-SD3 at 30 and 35 cycles. All above results suggested that IFI27 was down regulated by LMP2A or EBV.


2A 2B Neo 2A 2B Neo 2A 2B Neo 2A 2B Neo
20
25
30
35
IFI27
GAPDH
A


KMH2 K-EBV
KMH2 K-EBV
KMH2 K-EBV
KMH2 K-EBV
20
25
30
35
IFI27
GAPDH
B


L591-SD3 L591
L591-SD3 L591
L591-SD3 L591
L591-SD3 L591
20
25
30
35
IFI27
GAPDH
C

4.3 Discussion.

Classical Hodgkin's lymphoma is characterized by the presence of multinucleated, malignant HRS cells, which constitute only a minority of the tumour cells (Kuppers *et al.*, 2002). HRS cells are presumed to derive from germinal centre B cells that have lost their expression of immunoglobulin (Ig) and other B-cell-specific characteristics (Kuppers *et al.*, 2002; Cossman *et al.*, 1999; Marafioti *et al.*, 2000). It has been estimated that nearly half of all HL contain EBV DNA (Kuppers *et al.*, 2002; Khan and Coates, 1994; Pallesen *et al.*, 1991; Wu *et al.*, 1990) and the consistent detection of LMP2A transcripts in both tumour cells and latently infected B cells in vivo suggests that LMP2A plays an important role in viral persistence and in the development of HL. In this study, the impact of LMP2A and of LMP2B on global gene expression was investigated in KMH2 HL cells. Many genes found in this study have been previously reported in association with HL or EBV, these were mainly oncogenes or genes involved in signal transduction. Compared with LMP2B, LMP2A altered a greater proportion of genes involved in signal transduction and transcription; confirming previous studies that show the N-terminus of LMP2A, particularly its ITAM to be important for signalling (Longnecker, 2000). LMP2B was reported to play a role in cell adhesion and motility of epithelial cells (Allen *et al.*, 2005). In HL cells, LMP2B also altered a proportion of genes related to adhesion and motility.

HRS cells have lost the expression of most B-cell-specific genes, including the kinases Syk and Lyn and the adaptor molecule BLNK (Schwering *et al.*, 2003). Studies utilizing mice deficient in the BCR signalling molecules BLNK and Bruton's tyrosine kinase (BTK) have demonstrated that LMP2A utilizes these molecules for the effects on B cell development and survival (Engels *et al.*, 2001;

Merchant and Longnecker, 2002). These molecules are not only crucial for BCR signalling, but are also essential components of the signalling pathway (Engels *et al.*, 2001). In this study, many genes involved in BCR signalling, which were reported to be down regulated in HL were found to be up regulated by LMP2A (Shaffer *et al*, 2002; Portis *et al.*, 2004; Schwering *et al.*, 2003A; Schwering *et al.*, 2003B; Shen *et al.*, 2004; Feldhahn *et al.*, 2002; Wright *et al.*, 2003). These included *BLNK* and *BRDG,* which are docking proteins of downstream BCR signalling. They also included *IGKC/V* and *AICDA* which are involved in gene rearrangement; AICDA initiates somatic hypermutation (SHM) in B cells. *NEDD9* and *RGS3* regulate B cell adhesion, migration and immune responses. Taken together, these results suggest that LMP2A might use some components of the BCR signalling pathway and in doing so, maintain EBV latency, provide survival and anti-apoptotic signals for HRS cells, possibly transform B cells into HRS cells and allow survival of the transformed cells in the absence of a BCR. Thus, LMP2A might be important in HL because it selectively utilises some of the B cell signalling machinery.

AICDA was discovered as an APOBEC1 homolog with cytidine deaminase properties in stimulated B cell lines (Muramatsu *et al.*, 1999). AICDA is necessary for somatic hypermutation, class switch recombination and gene conversion. Inappropriate expression of cytidine deaminases might be tumorigenic. Consistent with this, inappropriate or down-regulated expression of cytidine deaminases was often found in tumors (Anant and Davidson, 2003). As abnormal SMH targets many proto-oncogenes *PIM1, PAX5, Rho/TTF* and *cMYC,* AICDA is regarded as a potential oncogene as well (Pasqualucci *et al.*, 2004). AICDA was constitutively expressed in GC B-cells and in GC-derived B-cell

lymphomas, including follicular lymphoma (FL), Burkitt lymphoma (BL) (Smit *et al.*, 2003; Greeve *et al.*, 2003), and chronic lymphocytic leukemia (Oppezzo *et al.*, 2003). Clonal Ig gene rearrangements had been detected in HRS cells identifying the majority of cases as B-cell-derived (Kuppers *et al.*, 1994). Evidence of SHM was found in the Ig V genes of major types, cHL and HLnlp (Kanzler *et al.*, 1996; Marafioti *et al.*, 2000). However, AICDA was consistently expressed in the L&H cells in HLnlp, but not in HRS cells in cHL cases (Greiner *et al.*, 2005). Although AICDA was initially identified as a gene upregulated by LMP2A, it was clear that this was only the case when LMP2A levels were higher, ie at 8 weeks post infection. In contrast, at two weeks post-infection, when LMP2A levels were low, AICDA was downregulated compared to control cells. Furthermore, AICDA was also downregulated in KMH2 cells infected with EBV and also EBV positive L591 cells compared with their counterparts. Thus it appears that 'physiological' levels of LMP2A might be actually associated with AICDA downregulation. Unfortunately, AICDA antibodies are not commercially available, immunoblotting with the anti-AICDA monoclonal antibody described by Greiner *et al.*, (2005) gave poor results. Therefore, it will still be necessary to confirm the RNA expression change described here at the protein level.

IFN-α mediates several biological responses, including inhibition of cell proliferation, antiviral reactions and immunoregulation (Stark *et al.*, 1998). These biological responses are initiated through the binding of IFN-α to cell-surface receptors, which activate signalling cascades that lead to changes in the transcription of target genes. Interferons exert their biological function mainly through the activation of interferon-stimulated genes (ISGs). IFI27 belongs to the family of small, IFN-α inducible genes of unknown function. It was first

described in 1993 and was found to be up-regulated in ~50% of breast carcinomas (Rasmussen *et al.*, 1993). IFI27 localises to the nuclear membrane (Martensen *et al.*, 2001). Recently, it was shown that STAT2-regulated expression of IFI27 was selectively enhanced by the chromatin remodelling factor BRG1 (Brahma-related gene 1) (Huang *et al.*, 2002). BRG1 has been implicated in regulating gene expression, cell-cycle control and tumourigenesis (Khavari PA *et al.*, 1993). IFI27 was shown by microassay analysis to be upregulated in PAL (Pyothorax-associated lymphoma) (Nishiu *et al.*, 2004). PAL is a unique lymphoma, which shows a strong association with EBV infection. Over-expression of IFI27 was also found in cell lines derived from PAL by Q-RT-PCR, but not in other lymphoid cell lines, Raji (EBV-positive BL cell line), BJAB (EBV-negative BL cell line), Namalwa (EBV-positive BL cell line), and LCL (EBV-positive lymphblatoid cell line). In this study, IFI27 was found to be down regulated by LMP2A, but not LMP2B. Unfortunately, these expression changes could not be confirmed at protein level since a suitable antibody was not available. Furthermore, down regulation of IFI27 was also observed in EBV infected KMH2 cells and L591 cells compared with their EBV-negative counterparts compared with its parental counterparts. However, how LMP2A or EBV downregulate IFI27 in HL cell lines and what the consequences of this downregulation are need to be further investigated.

In conclusion, this study shows the value of gene microarray technology to study altered gene expression in HL cells in response to LMP2 expression. LMP2A induced more marked effects on gene expression than LMP2B, particularly on genes with a transcriptional and signal transduction function. LMP2A might selectively utilise some components of the BCR signalling pathway to induce

cellular responses of relevance to the pathogenesis of HL. AICDA was downregulated in KMH2 cells expressing lower levels of LMP2A, KMH2 cells infected with EBV, and EBV positive L591 cells. In contrast, later passage cells expressing higher levels of LMP2A upregulated AICDA. Thus it appears that 'physiological' levels of LMP2A are important in determining the gene expression consequence of this protein. LMP2A might maintain EBV latency and provide its survival signals for HRS cells through down regulating IFI27. The relationship between LMP2 and cellular gene expression levels in HL are only descriptive at this stage, but have identified a number of areas for further study.

CHAPTER FIVE: IDENTIFICATION OF CELLULAR BINDING PARTNERS OF LMP2A AND LMP2B

5 Identification of cellular binding partners of LMP2A and LMP2B

5.1 Introduction

LMP2A and LMP2B are expressed in the majority of EBV-associated malignancies and also in the B cells of asymptomatic carriers suggesting they are important not only to the development and maintenance of the transformed phenotype but also to viral persistence.

LMP2 has been shown to alter cellular signalling; it can inhibit the switch from latent to lytic cycle in B cells by blocking BCR signals (Miller *et al.*, 1993; Miller *et al.*, 1995; Longnecker 2000), particularly the LMP2A N-terminus, which encodes a 119-amino-acid N-teminal cytoplasmic domain (LMP2A-N) is unique to LMP2A. It contains an immunoreceptor tyrosine-based activation motif (ITAM) (Fruehling *et al.*, 1996; Fruehling and Longnecker, 1997; Fruehling *et al.*, 1998; Longnecker, 2000) and has been shown to be responsible for blocking BCR-stimulated calcium mobilisation, tyrosine phosphorylation and activation of the EBV lytic cycle in B cells (Miller *et al.*, 1995).

In vivo models of EBV latent infection show that LMP2 can provide a survival signal to primary B lymphocytes. LMP2 is also important for virus persistence in the human host (Caldwell *et al.*, 1998; Caldwell *et al.*, 2000). It has already been reported that LMP2 associates with LMP1, Src family tyrosine kinases, the Syk tyrosine kinase, Nedd4 ubiquitin ligases, and with at least four unidentified cell proteins, several of which are tyrosine phosphorylated (Stewart *et al.*, 2005; Longnecker, 2000; Dykstra *et al.*, 2001; Ikeda *et al.*, 2000; Fukuda *et al.*, 2004; Fukuda *et al.*, 2005; Ikeda *et al.*, 2003; ingham *et al.*, 2005). LMP2A interacts with the extracellular signal-regulated kinase 1 (ERK1) mitogen-activated protein kinase (MAPK), and might contribute to LMP2A-induced activation of JUN,

MAPK kinase and the PI3K/Akt pathway (Chen *et al.*, 2002; Swart *et al.*, 2000; Fukuda *et al.*, 2004). However, most of these interactions were defined by methods such as immunoprecipitation, and are likely to represent only a fraction of the interactions that are important for the function of LMP2. To date, there has been no large scale systematic study to identify LMP2 interacting cellular proteins.

Mass spectrometry techniques have been developed for large-scale screening to identify interactions, but the nonquantitative nature of mass spectrometry restricts the ability to detect specific alterations in the composition of a protein complex. Mann and his colleagues established the SILAC technology (Ong *et al.*, 2002; Ong *et al.*, 2003a; Blagoev *et al.*, 2003; Foster *et al.*, 2003;Ong *et al.*, 2003b). Recently there have been many reports that document its successful use in identifying protein-protein interactions (Zhang *et al.*, 2006; Romijn *et al*, 2005; Gruhler *et al.*, 2005; Foster *et al.*, 2006; Amanchy *et al.*, 2005; Everley *et al.*, 2005). In this study, SILAC combined with LC-MS/MS analysis was used to identify potential LMP2A and LMP2B interacting proteins.

Aims of this research:

1 To generate His-tagged constructs of LMP2A, LMP2B and LMP2A-N.

2 To generate LMP2A and LMP2B expressing HEK cells.

3 To identify LMP2-interacting cellular proteins in these cells using the SILAC method.

5.2 Results

5.2.1 Production of plasmids

5.2.1.1 PCR for LMP2A, LMP2B and LMP2A-N

PCR was used to amplify LMP2A, LMP2B and LMP2A-N (Table 5.1) using pSG5-LMP2A plasmid DNA as template, which contains full length LMP2 gene (Gifted from Dr CW Dawson, university of Birmingham). Primers were designed to include the two cloning site sequences of pCDNA4 max/His A vector (see section 2.7.1), KpnI (GGTACC) and Xbal (TCTAGA). PCR conditions were optimised for different concentrations of $MgCl_2$ (1.0mM, 1.5mM, 2.0mM, and 2.5mM). For LMP2A, there was no difference among different concentrations of $MgCl_2$; for LMP2B, there were strong signals with 2.0mM and 2.5mM of $MgCl_2$, but no signal with 1.0 and 1.5 mM of $MgCl_2$. For LMP2A-N-terminus, the signal with 2.5mM of $MgCl_2$ was weaker than the others (Figure 5.1).

Table 5-1 Key to the nomenclature of plasmids and their products

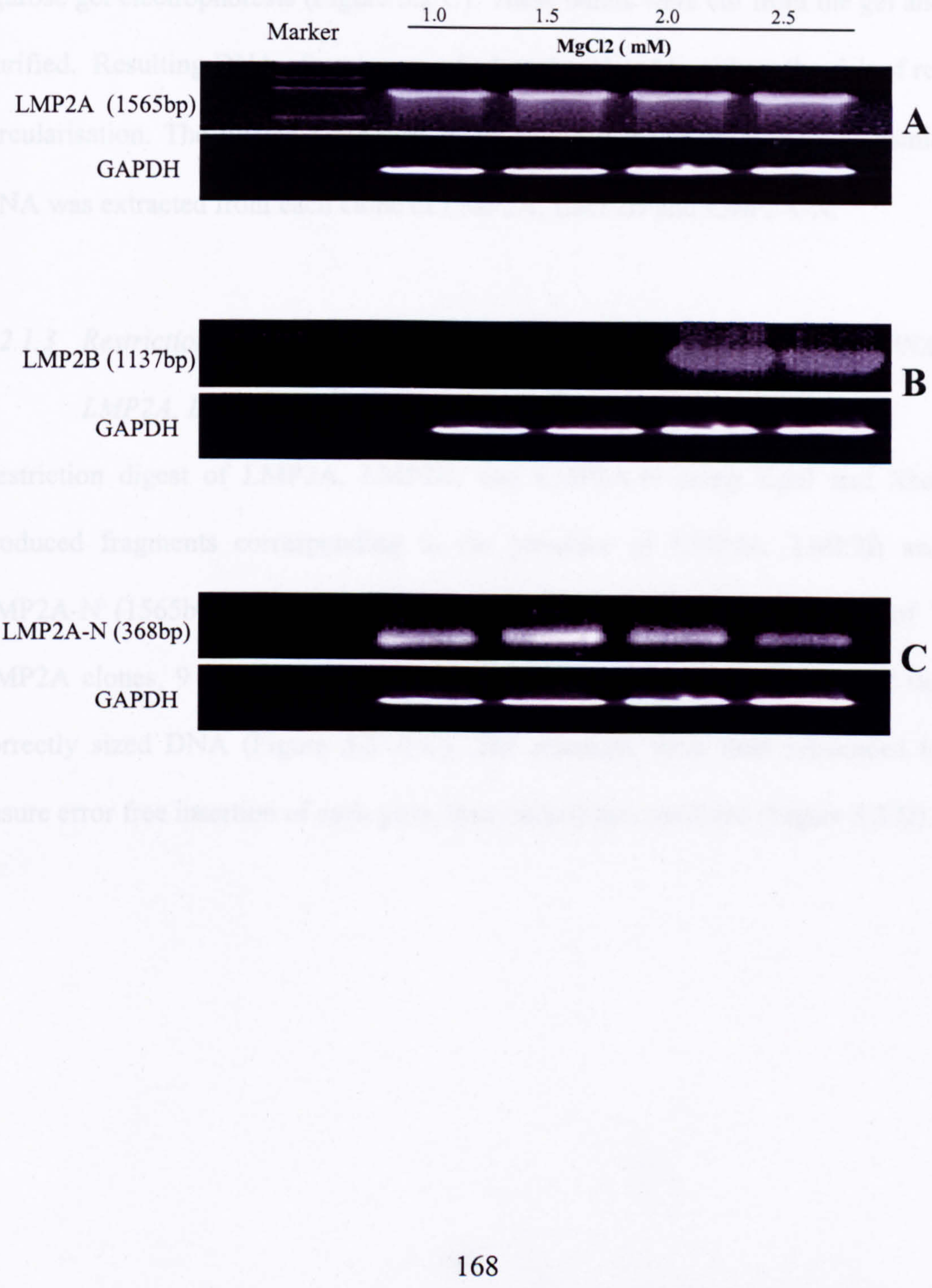
Plasmids	pcDNA4-LMP2A	pcDNA4-LMP2B	pcDNA4-LMP2A-N-terminus	pcDNA4 Max/His A
Short name	LMP2A	LMP2B	LMP2A-N	vector
Size of PCR products	1565bp	1137bp	368bp	5.3kb
Transfected cell lines	293-2A	293-2B	293-2A-N	Zeo
Size of proteins	54KDa	38KDa	16KDa	-

Figure 5-1 PCR results for LMP2A, LMP2B and LMP2A-N

(A) The LMP2A PCR product was 1565bp in size. The intensity was unaffected by different concentrations of $MgCl_2$.

(B) The LMP2B PCR product was 1137bp in size. There were strong signals with 2.0mM, 2.5mM $MgCl_2$ and no signal with 1.0mM and 1.5 mM $MgCl_2$.

(C) The LMP2A-N PCR product was 368bp in size. The signal with 2.5mM $MgCl_2$ was weaker than the others.


Marker
1.0
1.5
2.0
2.5
MgCl2 (mM)
LMP2A (1565bp)
GAPDH
A
LMP2B (1137bp)
GAPDH
B
LMP2A-N (368bp)
GAPDH
C

5.2.1.2 Restriction digest of LMP2A, LMP2B, LMP2A-N and Vector DNA

100 µl of each PCR product of LMP2A, LMP2B and LMP2A-N was purified and eluted in 50µl elution buffer. 3µg each of LMP2A, LMP2B or LMP2A-N DNA were digested with enzymes, KpnI and Xbal at room temperature overnight. Following restriction digest of DNA, strong and intense bands were observed at 1565bp for LMP2A, 1137bp for LMP2B and 368bp for LMP2A-N (Figure 5.2 A, B). The target vector, 5.3 kb was restricted with KpnI and Xbal, and resolved by agarose gel electrophoresis (Figure 5.2 C). These bands were cut from the gel and purified. Resulting DNA of vector was dephosphorylated to reduce the risk of re-circularisation. The ligated DNA was transformed into DH5α bacteria. Plasmid DNA was extracted from each clone of LMP2A, LMP2B and LMP2A-N.

5.2.1.3 Restriction digest of Mini-prepare and Bulk-prepare plasmid DNA, LMP2A, LMP2B and LMP2A-N

Restriction digest of LMP2A, LMP2B, and LMP2A-N using KpnI and Xbal produced fragments corresponding to the presence of LMP2A, LMP2B and LMP2A-N (1565bp, 1137bp and 368bp respectively) and 5.3kb vector. 6 of 7 LMP2A clones, 9 of 10 LMP2A-N clones and 8 of 10 LMP2B clones had the correctly sized DNA (Figure 5.3 A-C). The plasmids were then sequenced to ensure error free insertion of each gene, then bulked and restricted (Figure 5.3 D).

Figure 5-2 Agarose gel electrophoresis following restriction digest of LMP2A, LMP2B, LMP2A-N and vector DNA

DNA was digested by two restriction enzymes, KpnI and Xbal.

(A) There were multiple bands for LMP2A, but the strong and intense band was at 1565bp. A clear single band was for LMP2A-N (368bp).

(B) Clear single bands were present for LMP2B (1137bp).

(C) The bands of 5.3kb in size were for the vector.

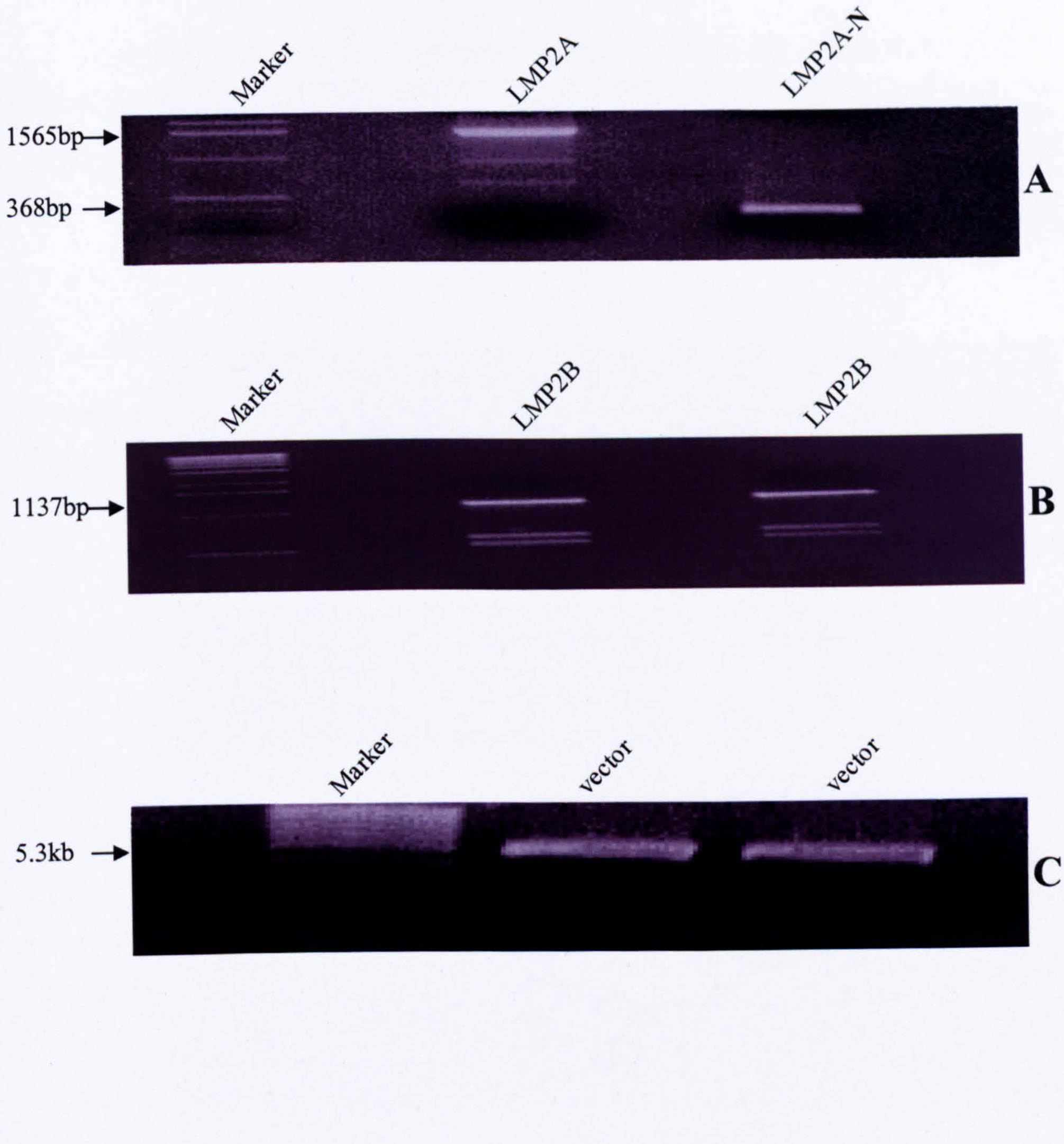

Marker
LMP2A
LMP2A-N
1565bp
368bp
A
Marker
LMP2B
LMP2B
1137bp
B
Marker
vector
vector
5.3kb
C

Figure 5-3 Analysis of restriction digest of Mini-prepare and Bulk-prepare plasmids, LMP2A, LMP2B and LMP2A-N

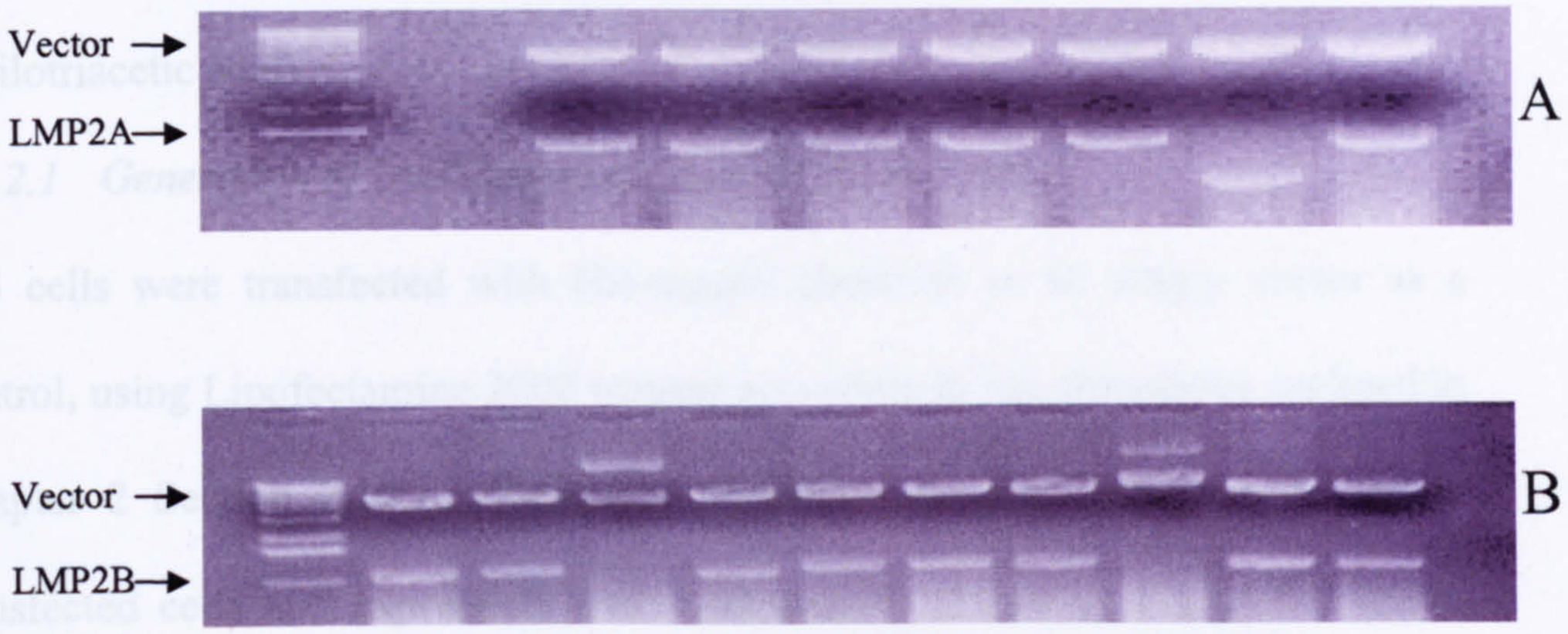
LMP2A, LMP2B and LMP2A-N plasmids were digested with KpnI and Xbal.

(A) 6 of 7 clones showed the correctly sized LMP2A insert (1565bp).

(B) 8 of 10 clones showed the correctly sized the LMP2B insert (1137bp).

(C) 9 of 10 clones showed the correctly sized LMP2A-N insert (368bp).

(D) Restriction digestion of the bulked plasmids.


Vector
LMP2A
A
Vector
LMP2B
B
Vector
LMP2A-N
C
Marker
LMP2A
LMP2A-N
Marker
LMP2B
D
Vector
D

5.2.2 Expression of LMP2 in HEK293 cells

Various techniques were tried to transfect HL cell lines with the His-tagged versions LMP2 plasmids generated above was unsuccessful, so they were used to transfect HEK293 cells, which are much more tolerable than HL cells. The Tag was used as a target to detect the expression of LMP2 in the transfected cells, but also as a bait for the purification of LMP2 proteins with Ni-NTA (nickel nitrilotriacetic acid).

5.2.2.1 Generation of LMP2-expressing HEK293 cell lines

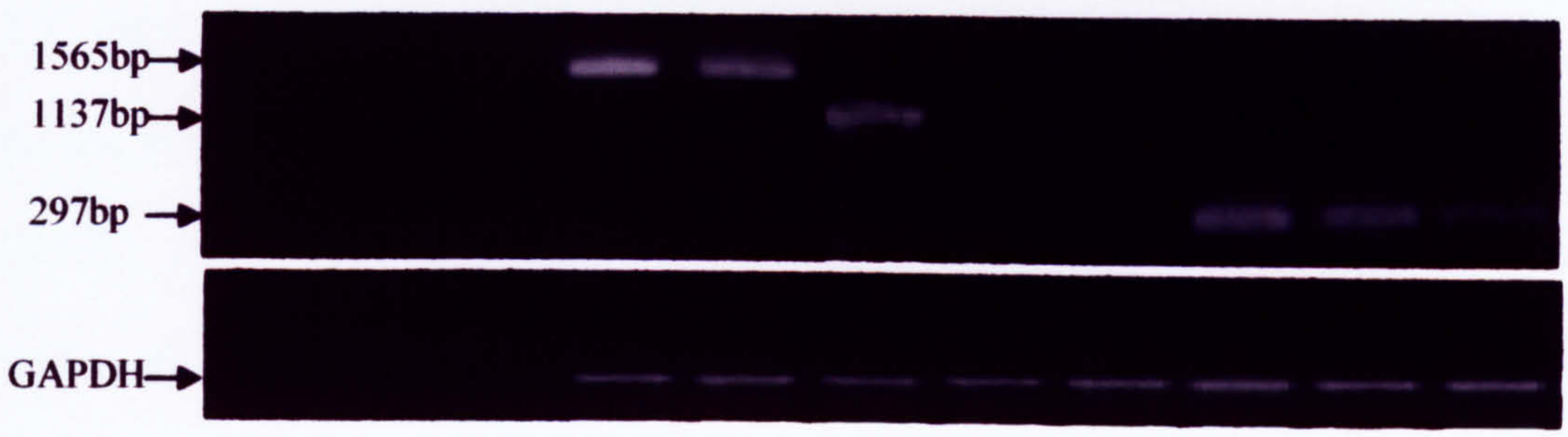
293 cells were transfected with His-tagged plasmids or an empty vector as a control, using Lipofectamine 2000 reagent according to the procedures outlined in Chapter 2 Section 2.10.1. Zeocin (500ug/ml) was used to select the stably transfected cells and expression was confirmed by RT-PCR. Figure 5.4 shows expression of LMP2A, LMP2B and LMP2A-N in each cell line. Immunofluorescence was used to confirm protein expression. Cells grown on Teflon-coated slides were stained using anti-His antibody (Upstate, 1:200) and Bala serum (1:50) (Figure 5.4). Control cells gave only a low background level of staining, whereas 293-LMP2A cells showed staining of perinuclear and cytosolic vesicles with both reagents. 293-2B and 293-2A-N cells were also stained with either reagent. However, 293-2B cells showed 100% positivity with Bala serum with strong, bright vesicular staining, but only a few cells showed perinuclear staining with His-antibody which was colocalised with the brightest staining of the Bala reagent. Only about 10% of 293-2A-N cells showed positive staining with both reagents (data not shown).

Figure 5-4 Confirmation of expression of LMP2A, LMP2B and LMP2A-N in transfected cell lines

(A) HEK293 cells transfected with His-tagged plasmids were subjected to RT-PCR to confirm expression from each construct. Full length LMP2A (1565bp) and LMP2B (1137bp) were amplified. Furthermore, LMP2A specific primers were designed that would specifically amplify a sequence unique to LMP2A (279bp) and were used to detect LMP-2A-N expression. Both 293-Zeo and K-Neo cells showed no signal for LMP2A. X50-7 was used as a positive control. GAPDH PCR was performed to ensure RNA integrity.

(B) Immunofluorescence staining showed that the 293-2A cells had strong, bright and vesicular staining with anti-His antibody and Bala serum, which was similar between the two reagents. 293-2B cells were also stained with both Bala serum and anti-His antibody. However, whereas all 293-2B cells were positive with Bala serum with strong, bright vesicular staining, only a few cells showing perinuclear staining were stained with the anti-His antibody which was colocalised with the brightest staining of the Bala reagent. 293-Zeo cells were used as a negative control and only showed low levels of background staining with either reagent.

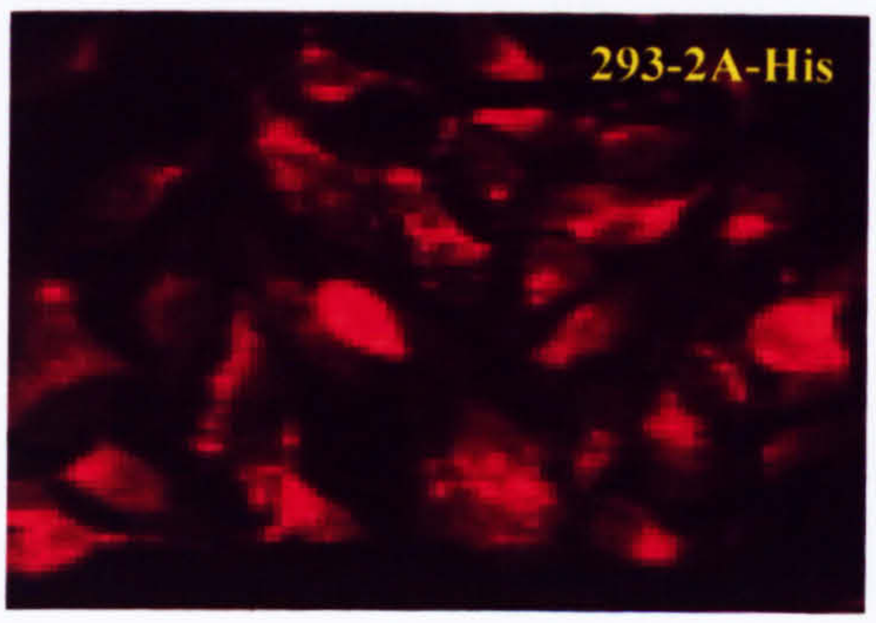
A

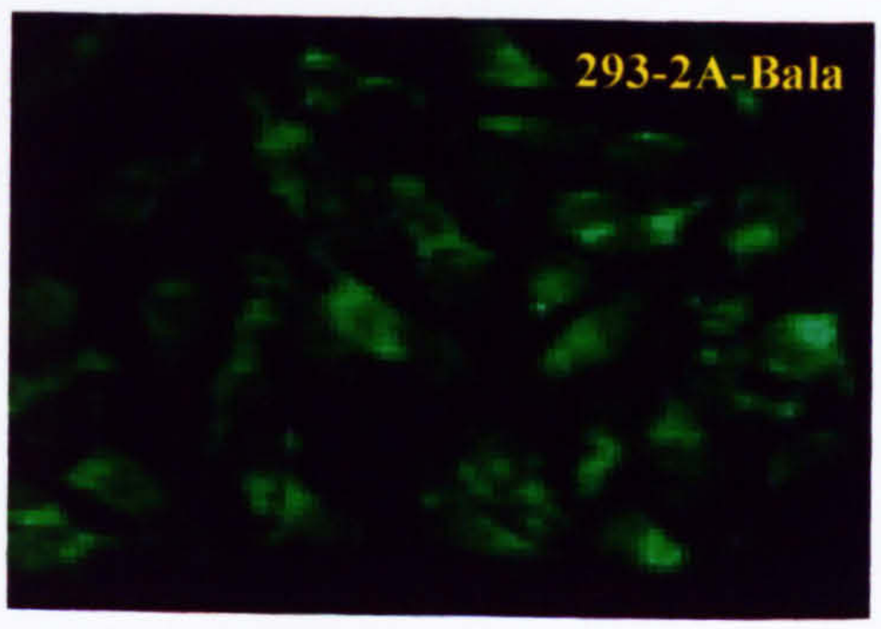

Marker
Negative
X50-7
293-2A
293-2B
293-Zeo
K-Neo
K-2A
293-2A
293-2A-N
1565bp
1137bp
297bp
GAPDH

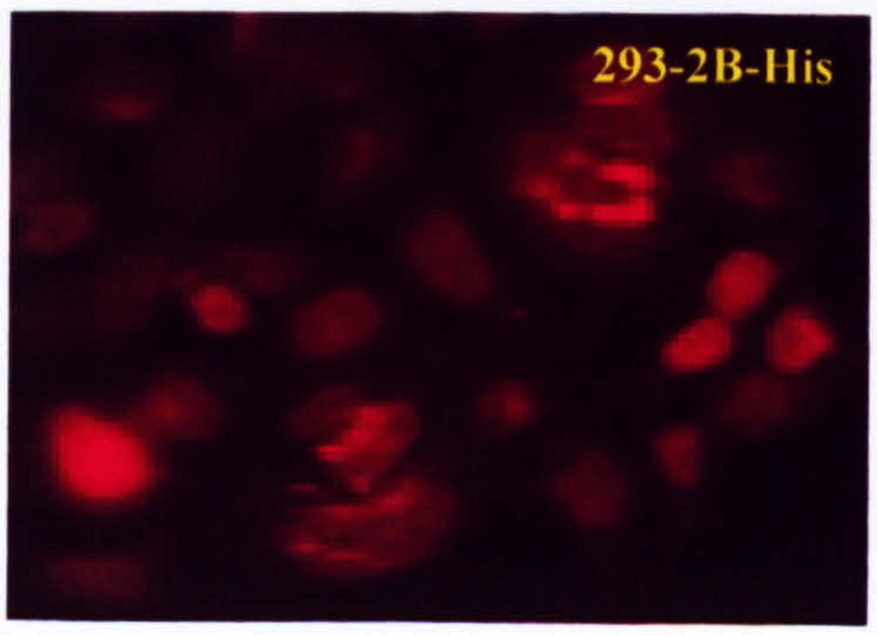
B

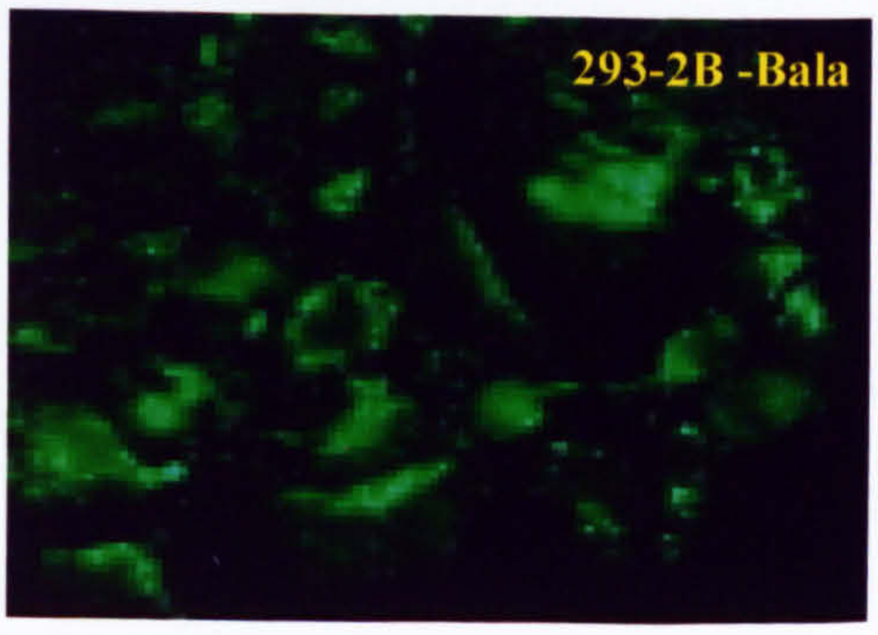

293-Zeo-His


293-Zeo-Bala


293-2A-His


293-2A-Bala


293-2B-His

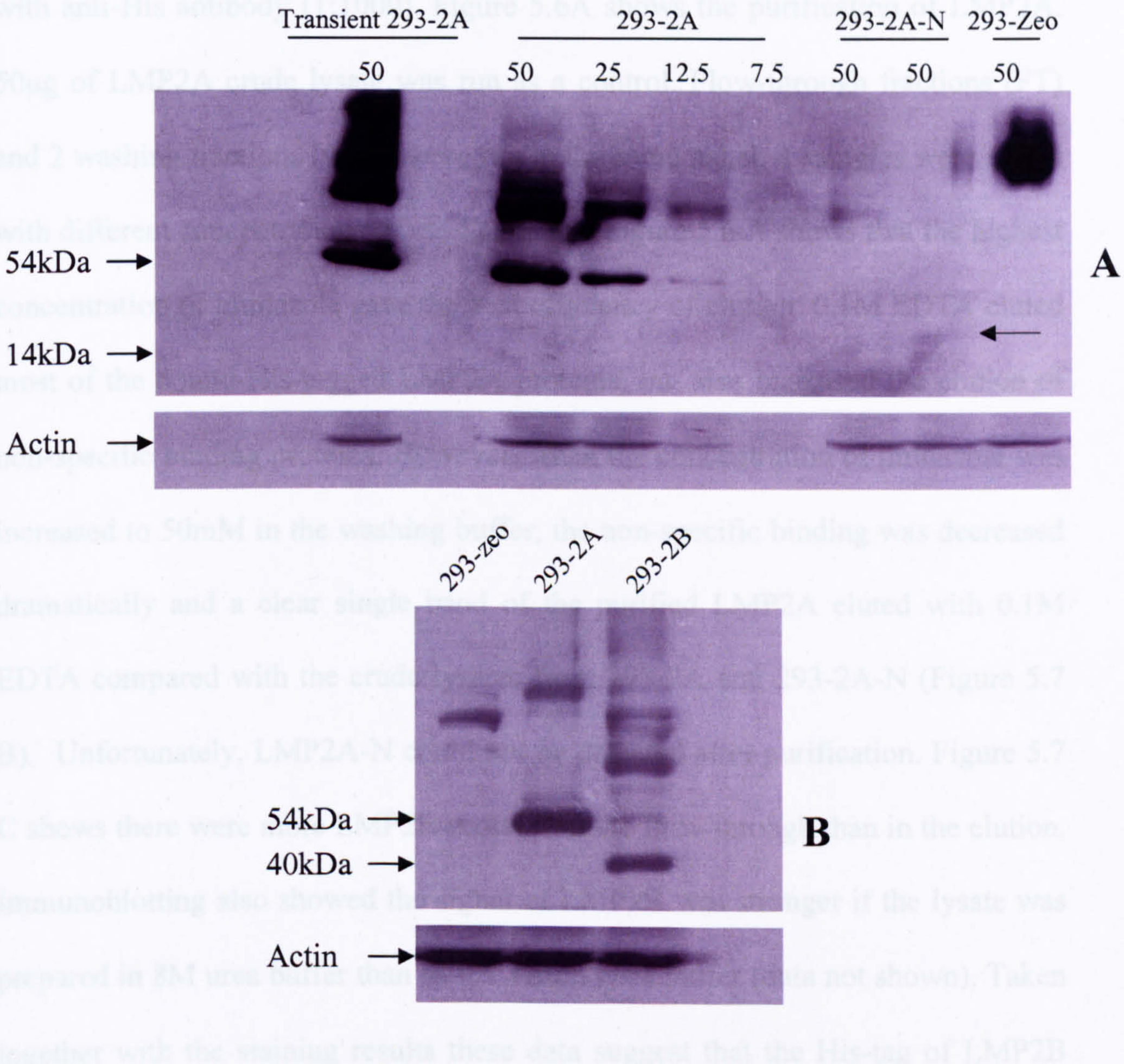

293-2B -Bala

To establish that transfected HEK293 cell lines were expressing full length tagged versions of LMP2A, LMP2B or LMP2A-N, protein expression was investigated by immuno-blotting (Section 2.4.2). Protein lysates were prepared with 1% Triton 100 (Sigma) and exposed to anti-His antibody (Upstate, 1:1000) and goat anti-mouse HRP secondary antibody (Dako, 1:1000). A series of concentrations from 293-2A cells (50μg, 25μg, 12.5μg and 7.5μg) were tested alongside 50μg of protein from parental 293 cells and from 293-2A-N cells. 50μg of protein from 293 cells transiently transfected with LMP2A were used as a positive control. Figure 5.5 A shows that full length LMP2A (54kDa) could be detected in 293-2A cells down to 12.5μg of protein. A very faint signal (14kDa) was detected from one clone of LMP2A-N cells. Figure 5.5 B shows the detection of LMP2B at the expected molecular weight (40kDa). It was observed that the signal for LMP2B was stronger if lysates of 293-2B cells were prepared using 8M Urea buffer instead of 1% Triton-100 (data not shown).

Figure 5-5 Expression of LMP2A and LMP2B in 293 cells detected by immunoblotting

(A) A series of protein concentrations from 293-LMP2A cells (50μg, 25μg, 12.5μg and 7.5μg) along with the 50μg of protein from 293-Zeo cells and from two clones of 293-2A-N cells. 50μg of protein from transiently transfected 293 cells with LMP2A was used as a positive control. The 54kDa LMP2A protein could be detected in 293-2A cells down to 12.5μg of protein. As expected, 293-Zeo cells showed no signal for LMP2A. A very faint signal for LMP2A-N (14kDa) was detected from one clone (pointed by an arrow).

(B) The 40kDa LMP2B protein was detected in 293-2B cells.


Transient 293-2A
293-2A
293-2A-N
293-Zeo
50
50
25
12.5
7.5
50
50
50
54kDa
14kDa
A
Actin
293-zeo
293-2A
293-2B
54kDa
40kDa
B
Actin

5.2.3 Purification of LMP2 proteins with Ni-NTA

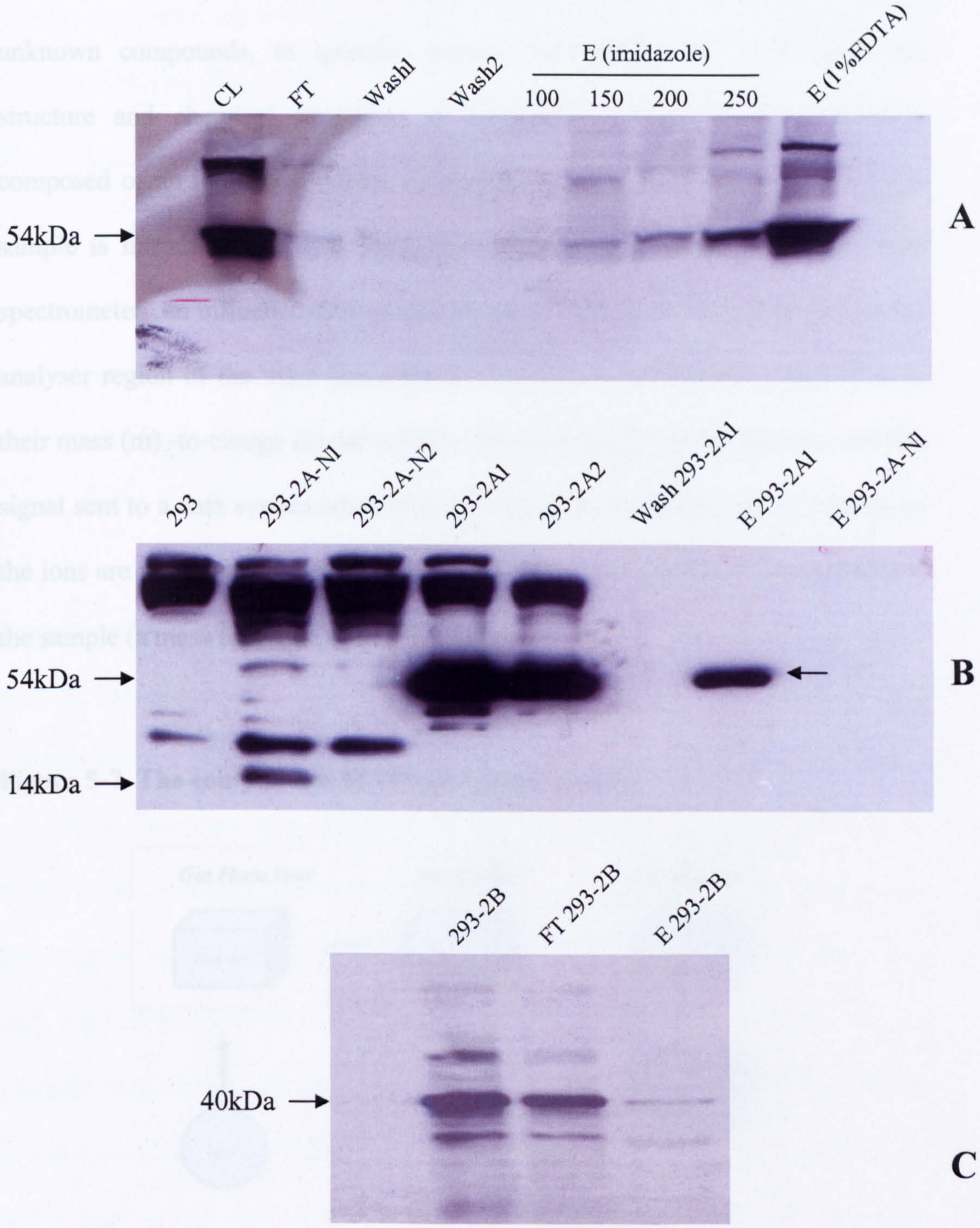
The His-tagged LMP2 proteins were purified as described in section 2.11. 1mg protein was incubated with 100µl of 50% v/v washed Ni-NTA beads. The proteins were eluted with 100µl pH 8.0 elution buffer with different concentration of imidazole (100mM, 150mM, 200mM, 250mM) and 0.1M EDTA (pH 8.0). 20µl of each eluted protein was loaded for western blotting and the membrane was blotted with anti-His antibody (1:1000). Figure 5.6A shows the purification of LMP2A. 50µg of LMP2A crude lysate was run as a control. Flow-through fractions (FT) and 2 washing fractions (wash) were run in the same panel. 4 samples were eluted with different concentrations of imidazole and Figure 5.6 A shows that the highest concentration of imidazole gave the best efficiency of elution. 0.1M EDTA eluted most of the bound His-tagged LMP2A proteins, but also increased the elution of non-specific binding proteins. However, when the concentration of imidazole was increased to 50mM in the washing buffer, the non-specific binding was decreased dramatically and a clear single band of the purified LMP2A eluted with 0.1M EDTA compared with the crude lysates from 293-2A and 293-2A-N (Figure 5.7 B). Unfortunately, LMP2A-N could not be detected after purification. Figure 5.7 C shows there were more LMP2B proteins in the flow-through than in the elution. Immunoblotting also showed the signal of LMP2B was stronger if the lysate was prepared in 8M urea buffer than in 1% Triton lysis buffer (data not shown). Taken together with the staining results these data suggest that the His-tag of LMP2B might have been partially hidden in the native conditions because of the conformation of the protein.

Figure 5-6 Purification of LMP2 proteins with Ni-NTA

(A) Purification of LMP2A. 50μg of LMP2A crude lysate (CL) was run as a control. Flow through (FT), wash1 and wash2 were run in the same panel. 4 samples were eluted with different concentrations of imidazole (100mM, 150mM, 200mM, and 250mM). The highest concentration of imidazole gave the best efficiency of elution. 0.1 M EDTA eluted most of the bound His-tagged LMP2A proteins, but also some non-specific proteins.

(B) A clear band of LMP2A (arrowed) was eluted with 0.1M EDTA when the concentration of imidazole was increased to 50mM in the washing buffer. No signal of LMP2A-N was shown after the purification.

(C) There were more LMP2B proteins in the FT than in the elution; this might be because the His-tag of LMP2B was partially hidden because of the protein conformation, so reducing the binding capacity of the His-tag.



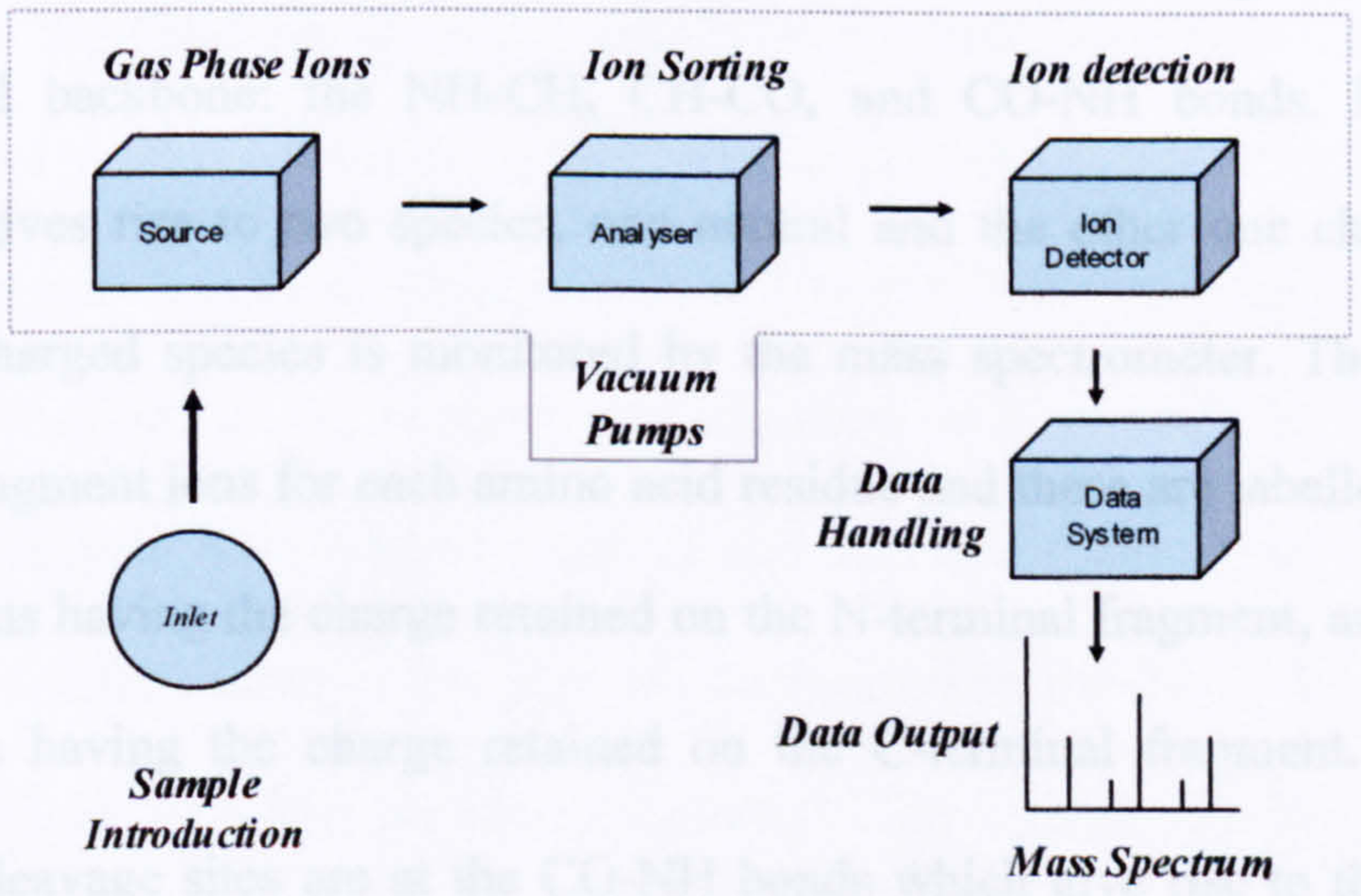
Key: CL=crude cell lysate FT=Flow-through fractions. Wash=washing fractions. ET=Elution fractions

5.2.4 Identification of LMP2A or LMP2B interacting proteins

5.2.4.1 Mass spectrometry

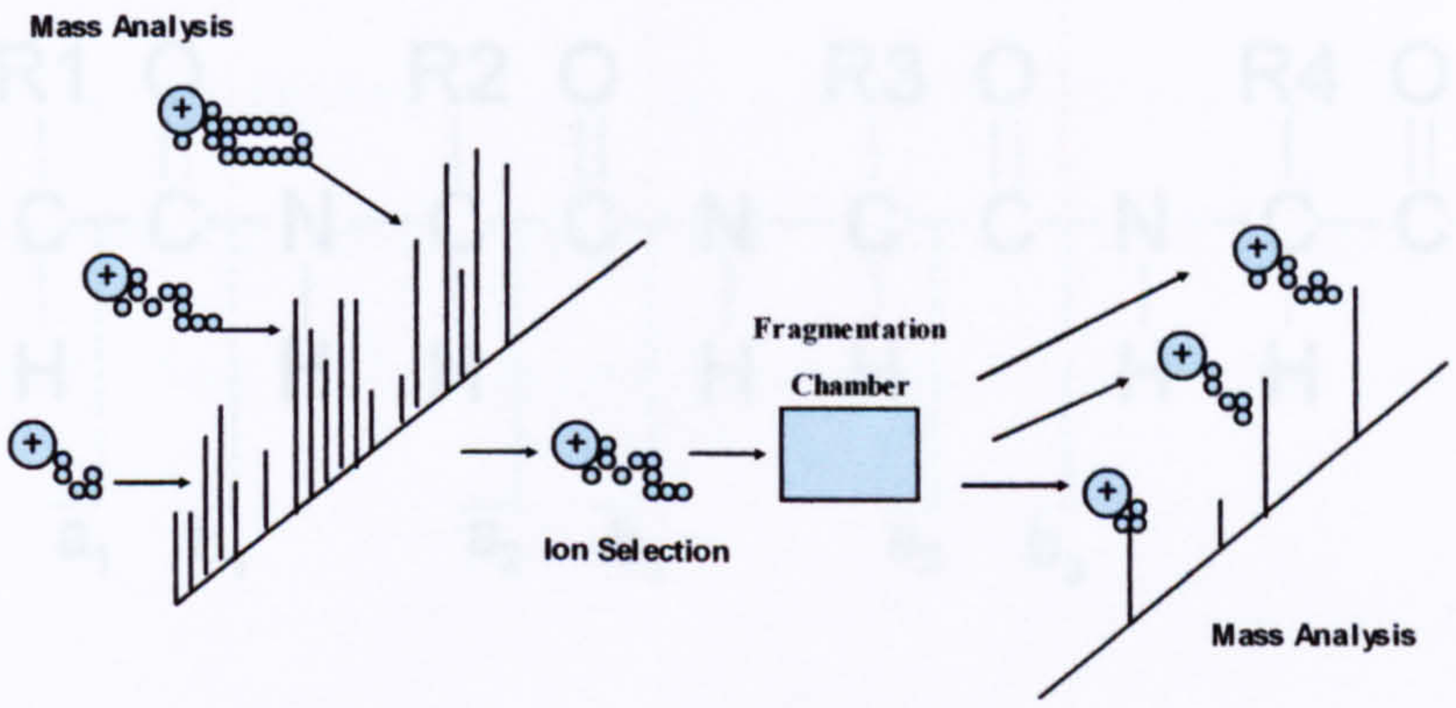
Mass spectrometry is a powerful analytical technique that is used to identify unknown compounds, to quantify known compounds, and to elucidate the structure and chemical properties of molecules. The mass spectrometer is composed of an ionisation source, an analyser, and a detector (Figure 5.7). The sample is introduced into the ionisation source to be ionised before the mass spectrometer can influence it in an electric field. These ions are extracted into the analyser region of the mass spectrometer where they are separated according to their mass (m) -to-charge (z) ratios (m/z). The separated ions are detected and this signal sent to a data system where the m/z ratios are stored and the m/z values of the ions are plotted against their intensities to show the number of components in the sample (a mass spectrum).

Figure 5-7 The components of a Mass Spectrometer



MS/MS is a coupling of two stages of mass analysis. From a mixture of ions in the source region, ions of a particular m/z value are selected in the first stage of mass analysis. These "parent" or "precursor" ions are fragmented and then the product ions resulting from the fragmentation are analyzed in a second stage of the mass analysis (Figure 5.8).

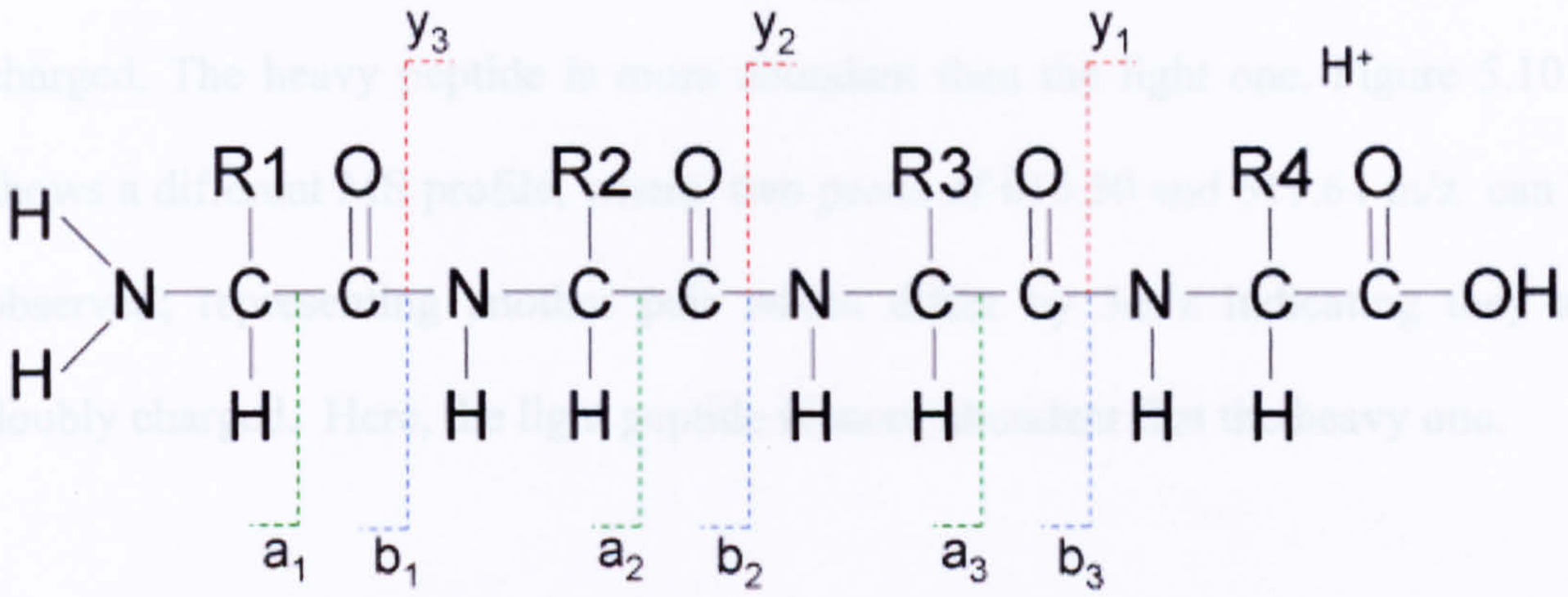
Figure 5-8 Schematic of MS/MS analysis



Fragmentation is a reaction whereby an ion is broken down into ions with smaller masses. The accepted nomenclature for fragment ions was first proposed in 1984 (Roepstorff and Fohlman, 1984) and subsequently modified in 1987 (Johnson *et al.,* 1987). There are three different types of bonds that can fragment along the amino acid backbone: the NH-CH, CH-CO, and CO-NH bonds. Each bond breakage gives rise to two species, one neutral and the other one charged, and only the charged species is monitored by the mass spectrometer. There are six possible fragment ions for each amino acid residue and these are labelled as the a, b, and c ions having the charge retained on the N-terminal fragment, and the x, y, and z ions having the charge retained on the C-terminal fragment. The most common cleavage sites are at the CO-NH bonds which give rise to the b and/or the y ions The **a** ions are often used as a diagnostic for b ions, such that **a-b** pairs are often observed in fragment spectra. The **a-b** pairs are separated by 28units,

the mass for the carbonyl, C=O. The precursor ion can be singly, doubly or triply charged. A doubly charged precursor can be fragmented into singly, doubly charged **a, b** and **y** ions. The fragment types showed below are the most common fragments observed (Figure 5.9).

Figure 5-9 Peptide Fragmentation Nomenclature a, b and y ions



5.2.4.2 Stable isotope labelling with amino acids in cell culture (SILAC) analysis

The SILAC method is outlined in detail in section 2.12. 293-2A, -2B or -Zeo cells were grown in the medium containing either [$^{13}C_6$]-arginine or normal [$^{12}C_6$]-arginine for at least 5 passages. Lysates were prepared from each treatment and mixed in a 1:1 ratio according to their protein concentrations, purified with Ni-NTA beads and then resolved on a 4–21% (wt/vol) SDS-PAGE gel. The entire lane was excised in ten sections and digested with trypsin for LC-MS/MS analyses. Samples were loaded, analysed and data was collected as described in section 2.12.5. The mass of the peptides determined in the initial MS analysis is shown in Figure 5.10. The peptides containing [$^{13}C_6$]-arginine residues are heavier by 6 Da than the corresponding peptides containing [$^{12}C_6$]-arginine residues, but they differ by 6 or 3 m/z (mass to charge ratio) as they are singly or doubly

charged. Figure 5.10 A shows a typical MS profile in which the retention time (RT) was 40.17 min. RT is correlating to identification of the eluting amino acid of a peptide or protein to a standard chromatogram of HPLC gradient. There is a clear peak of 626.40 m/z. The bottom panel shows an enlarged area around 626.40 m/z. Another peak of 620.24 m/z is observed, which could be the light isotopic partner. These two peaks are paired differently by 6m/z, indicating they are singly charged. The heavy peptide is more abundant than the light one. Figure 5.10 B shows a different MS profile, where two peaks of 616.50 and 619.64 m/z can be observed; representing another pair which differ by 3m/z indicating they are doubly charged. Here, the light peptide is more abundant that the heavy one.

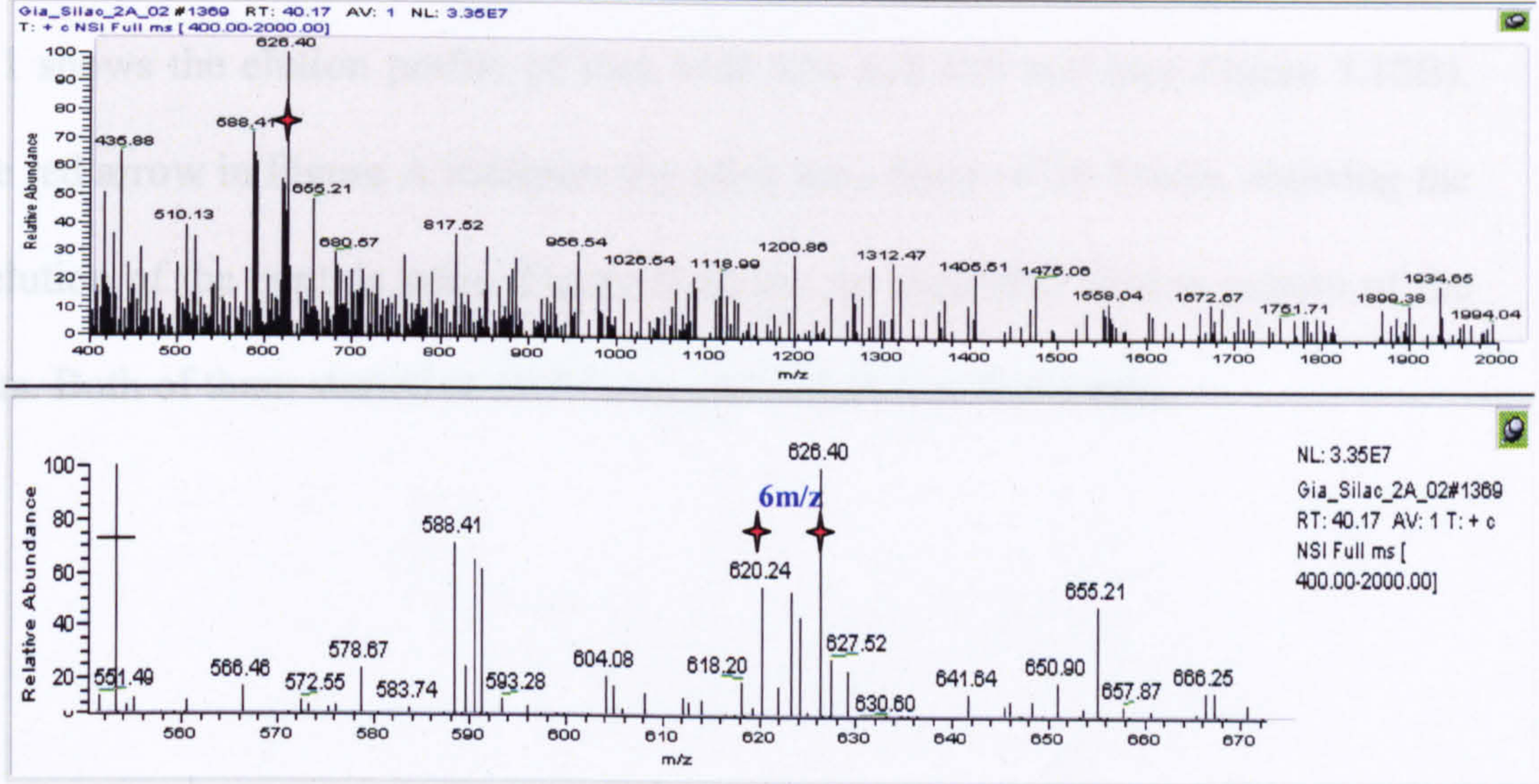
Figure 5-10 Mass spectrometric analysis

The peptides containing [$^{13}C_6$]-Arginine residues are heavier by 6Da than the peptides containing [$^{12}C_6$]-Arginine residues.

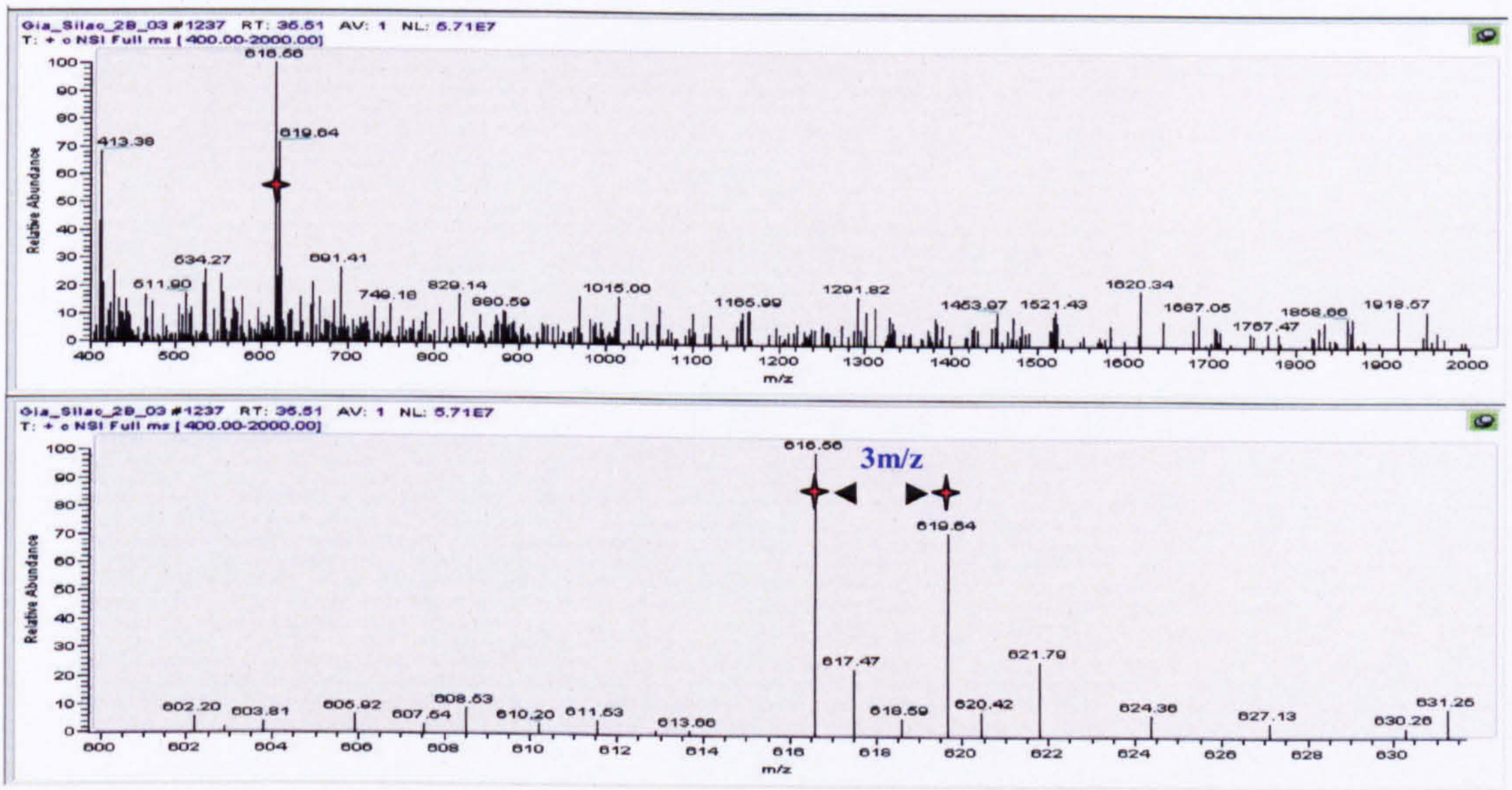
(A) The upper panel shows a typical MS profile, ranging from 400 to 2000 m/z, generated during the analysis. The retention time was 40.17 min. Between 600 and 700m/z, there was a clear peak of 626.40 m/z marked by a red cross. The lower panel shows an enlarged image of the area around 626.40m/z. Another peak of 620.24 m/z was found, which could be the light isotopic partner. These two peaks differed by 6m/z, which corresponds to the singly charged ions. The heavy peptide was more abundant than the light one.

(B) The upper panel shows another MS profile, ranging from 400 to 2000m/z, the retention time of which was 35.51 min. There were two clear peaks of 616.50 and 619.64 m/z, respectively marked by a red cross. Lower panel shows enlarged of this area. Another pair is repeated here which differ by 3m/z (doubly charged), in this example the light peptide was more abundant that the heavy one.

A


Gia_Silac_2A_02 #1369 RT: 40.17 AV: 1 NL: 3.35E7
T: + c NSI Full ms [400.00-2000.00]
Relative Abundance
m/z
626.40
588.41
435.88
655.21
510.13
680.67
817.52
956.54
1026.64
1118.99
1200.86
1312.47
1406.61
1475.06
1558.04
1672.67
1751.71
1899.38
1934.65
1994.04
6m/z
620.24
578.67
566.46
572.55
583.74
593.28
604.08
618.20
627.52
641.64
650.90
657.87
666.25
630.60
551.49
NL: 3.35E7
Gia_Silac_2A_02#1369
RT: 40.17 AV: 1 T: + c
NSI Full ms [
400.00-2000.00]

B


Gia_Silac_2B_03 #1237 RT: 36.51 AV: 1 NL: 5.71E7
T: + c NSI Full ms [400.00-2000.00]
Relative Abundance
m/z
616.66
619.64
413.38
634.27
611.90
691.41
749.18
829.14
880.59
1015.00
1165.99
1291.82
1453.97
1521.43
1620.34
1687.05
1767.47
1858.66
1918.57
3m/z
617.47
602.20
603.81
606.92
607.54
608.53
610.20
611.53
613.66
618.59
620.42
621.79
624.36
627.13
630.26
631.25

5.2.4.3 [$^{12}C_6$]- and [$^{12}C_6$]-peptides elute at the same time

Although arginine-containing peptides appeared in a light and heavy form separated by 6 Da, they co-elute because they are chemically indistinct. Relative quantification is performed from single peptide in the MS mode, relating the two monoisotopic peaks from peptides containing [$^{12}C_6$]-and [$^{13}C_6$]-Arginine. Figure 5.11 shows the elution profile of ions with 616 and 619 m/z (see Figure 5.10B). The red arrow in Figure A indicates the peak time point of 35.51min, showing the coelution of the peptide pairs. Figure B shows an expanded elution pattern of the pairs. Both of them started at 35.37 min and finished at 36.11 min.

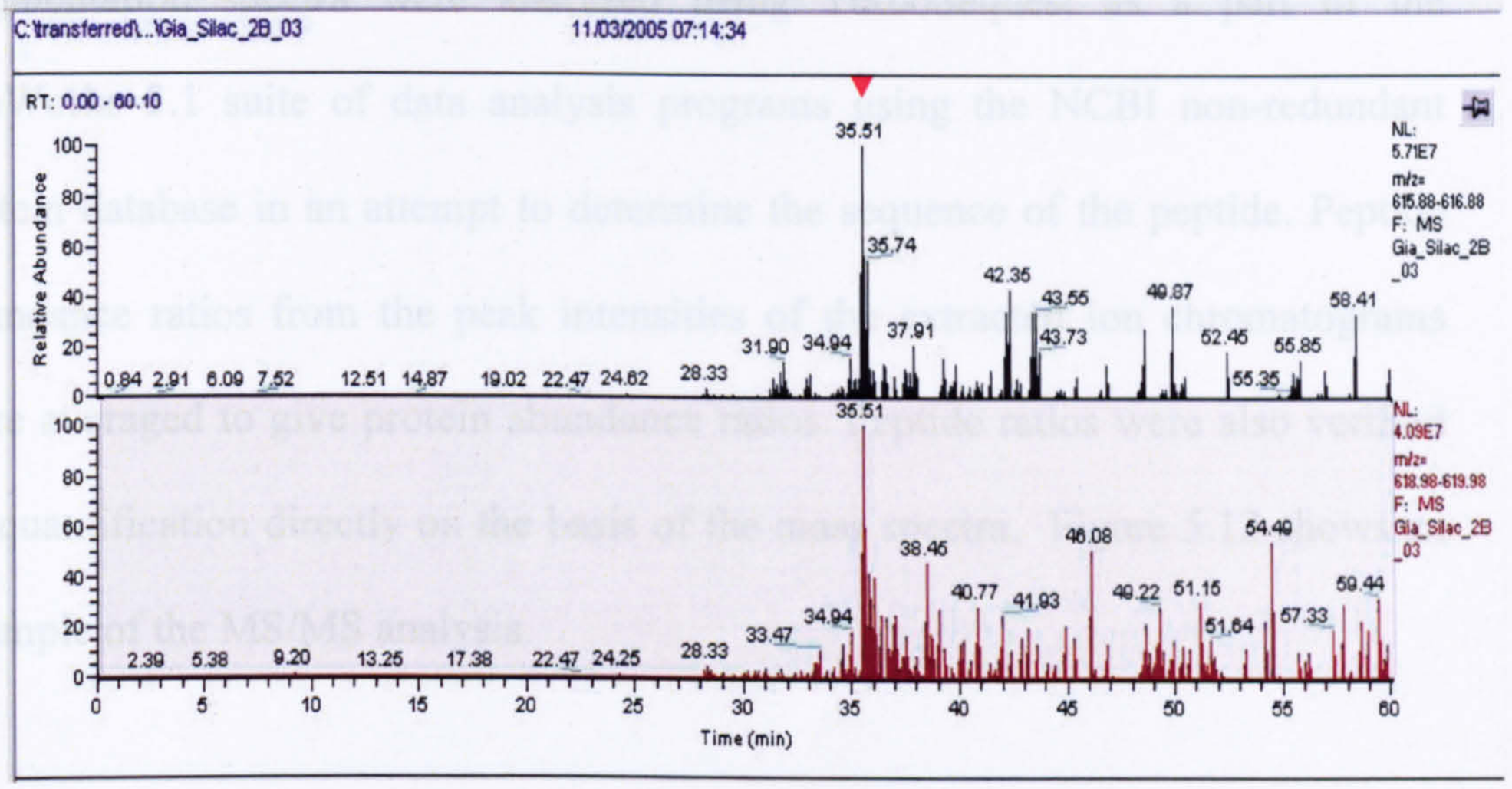
Figure 5-11 [$^{13}C_6$]- and [$^{12}C_6$]-peptides eluted at the same time

The elution profile of ions with of 616 m/z and 619 m/z is shown with a peak at 35.51 minutes. The X-axis is time (min) and Y-axis is relative abundance of peptides.

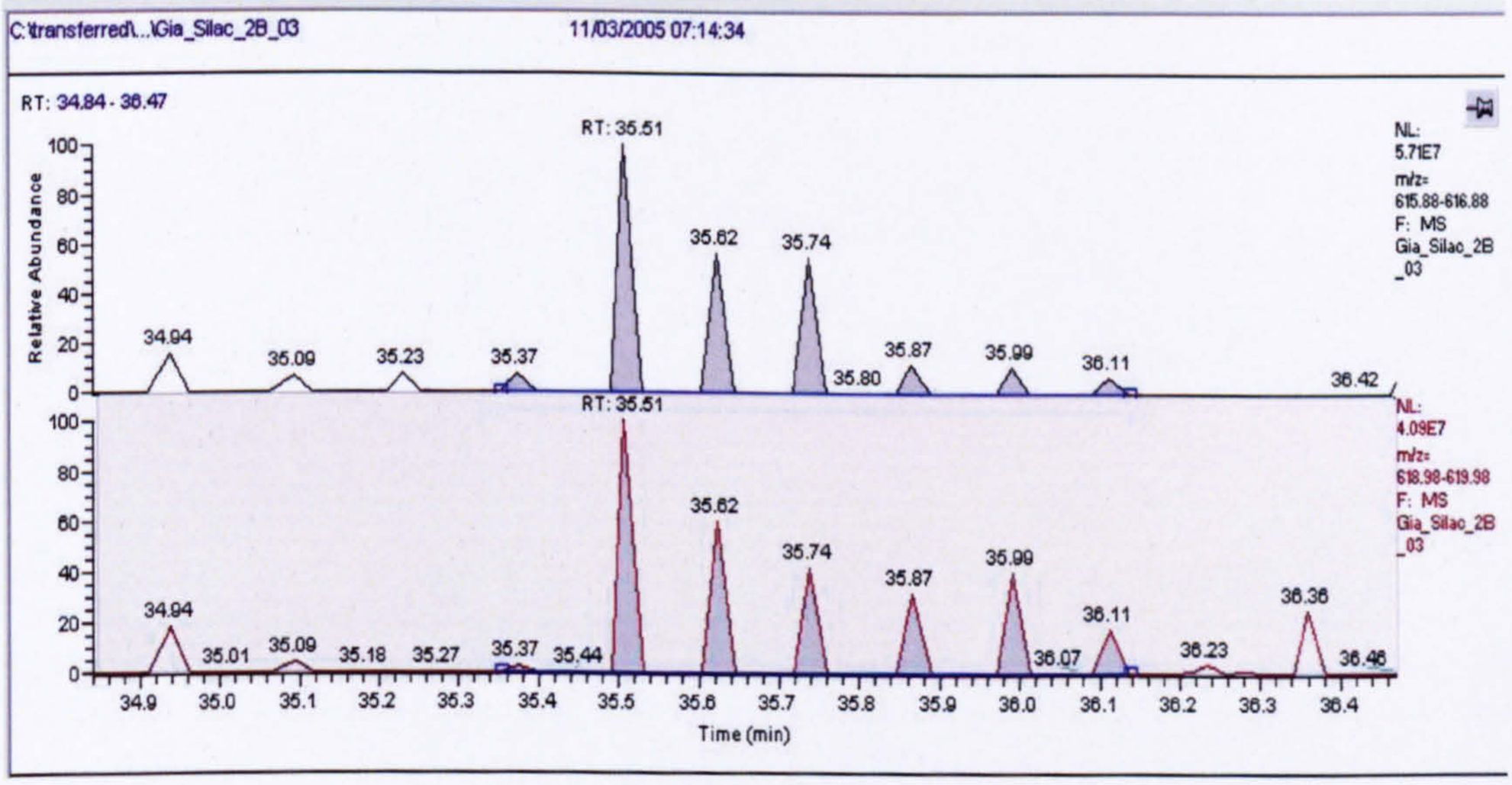
(A) The red arrow shows indicates the peak elution time 35.51min at which the light and heavy peptides eluted.

(B) An enlarged image of the peak elution time of the peptide pairs is shown. Both of them started from 35.37 and finished at 36.11 minutes.

A


C:\transferred\...\Gia_Silac_2B_03
11/03/2005 07:14:34
RT: 0.00 - 60.10
Relative Abundance
Time (min)
NL: 5.71E7 m/z= 615.88-616.88 F: MS Gia_Silac_2B_03
NL: 4.09E7 m/z= 618.98-619.98 F: MS Gia_Silac_2B_03
35.51
35.74
42.35
43.55
49.87
58.41
31.90
34.94
37.91
43.73
52.46
55.85
55.35
0.84
2.91
6.09
7.52
12.51
14.87
19.02
22.47
24.62
28.33
38.46
46.08
54.49
40.77
41.93
49.22
51.15
59.44
57.33
51.64
33.47
2.39
5.38
9.20
13.25
17.38

B


C:\transferred\...\Gia_Silac_2B_03
11/03/2005 07:14:34
RT: 34.84 - 36.47
Relative Abundance
Time (min)
NL: 5.71E7 m/z= 615.88-616.88 F: MS Gia_Silac_2B_03
NL: 4.09E7 m/z= 618.98-619.98 F: MS Gia_Silac_2B_03
RT: 35.51
35.62
35.74
34.94
35.09
35.23
35.37
35.80
35.87
35.99
36.11
36.42
35.01
35.18
35.27
35.44
36.07
36.23
36.36
36.46

5.2.4.4 MS/MS analysis

After the initial analysis of MS, the selected ions were subject to MS/MS analysis as a part of the automated analysis performed by the ion trap. The most abundant 5 ions were selected and analyzed sequentially after each full MS scan. The fragmentation spectra were analyzed using TurboSequest as a part of the BioWorks 3.1 suite of data analysis programs using the NCBI non-redundant protein database in an attempt to determine the sequence of the peptide. Peptide abundance ratios from the peak intensities of the extracted ion chromatograms were averaged to give protein abundance ratios. Peptide ratios were also verified by quantification directly on the basis of the mass spectra. Figure 5.12 shows an example of the MS/MS analysis.

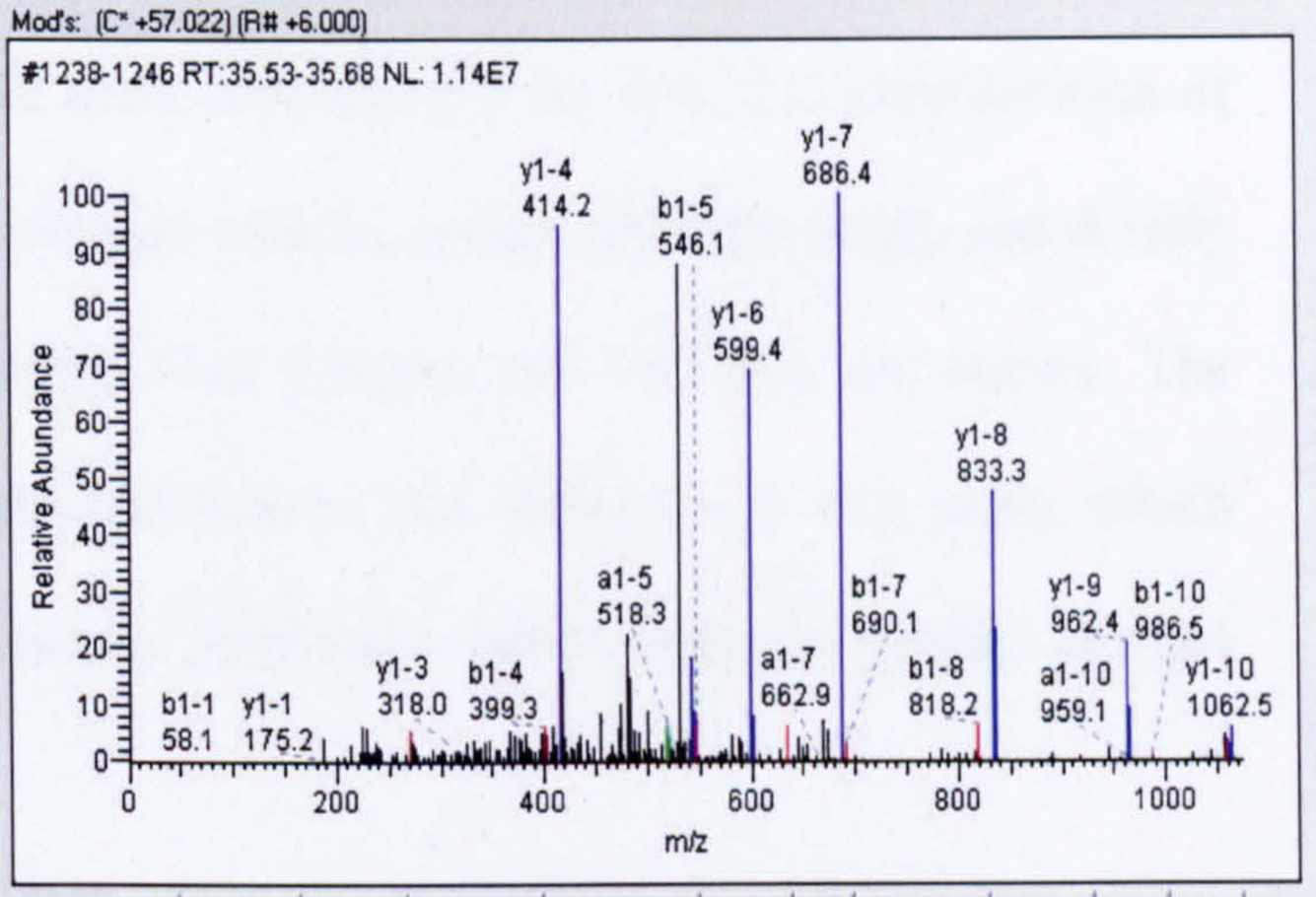
Figure 5-12 Evaluating the quality of the MS/MS analysis

This figure shows MS/MS analysis of fragmented ions generated from peptides shown in Figure 5.10. The a, b and y ions identified in the spectra are shown in green, red and blue, respectively. The top panel shows that 8/11 y-ions of the light peptide were detected and the bottom panel shows that 9/11 y-ions of the heavy peptide were observed. Most b-ions and some a-ions were also seen from the two peptides. The difference between the detected a-ions (green) and b-ions (red) in the two panels is 28 units. The fragmented ions in this example are singly charged (blue boxes). Between heavy ions (upper panel) and light ions (lower panel) shows they differ by 6 m/z as expected. The y ions are always the most abundant ions after fragmentation. The b ion fragments with no arginine are not different between the two panels.

Display the ion series for charge state: +1 Dta: Gia_Silac_2B_03.1238.1246.2 Precursor mass: 616.38 Mass type: Average

Mod's: (C* +57.022) (R# +6.000)

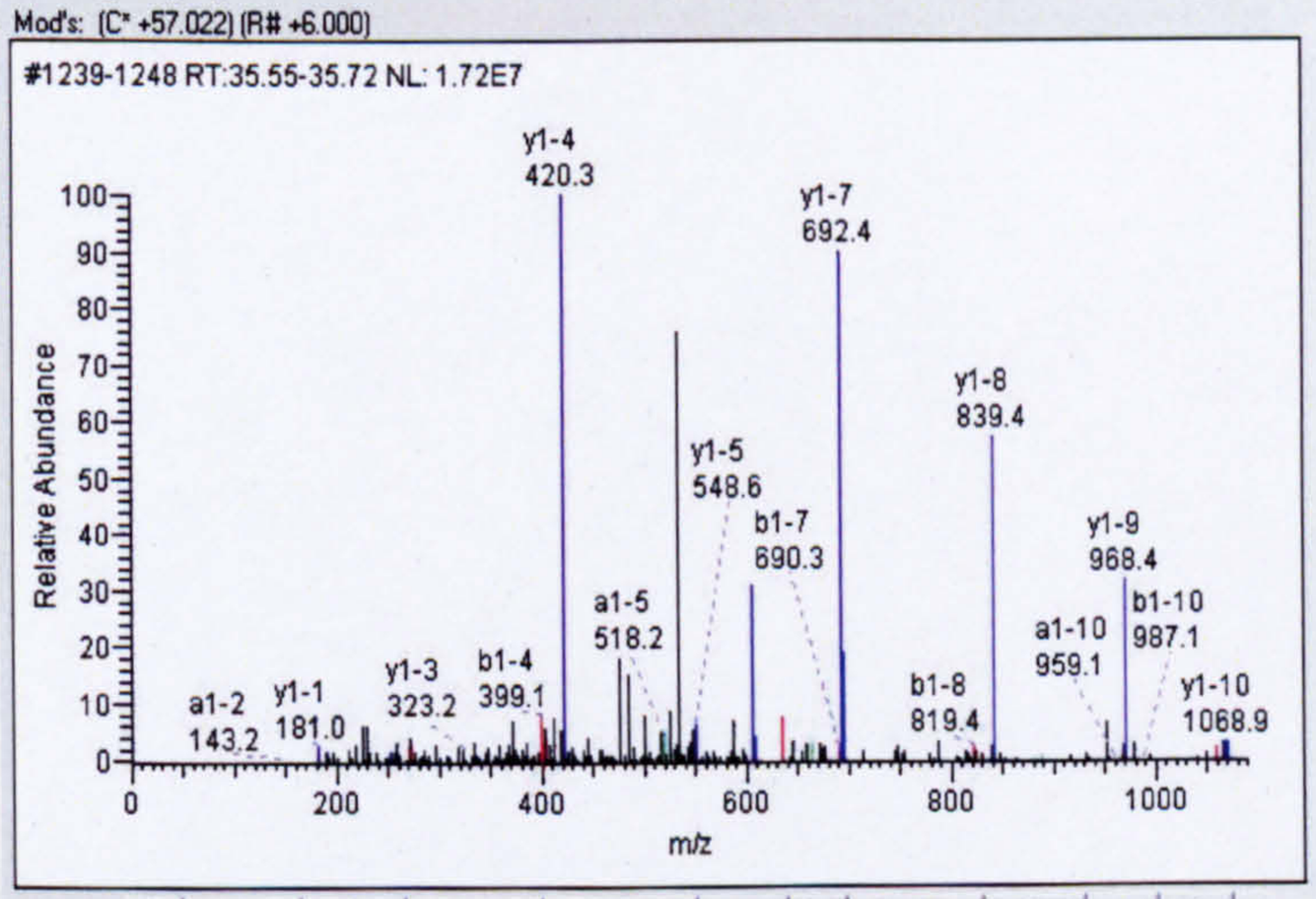
	AA	A Ions	B Ions	Y Ions	
1	G	30.05	58.06	-	12
2	I	143.21	171.22	1175.34	11
3	V	242.34	270.35	1062.18	10
4	E	371.46	399.46	963.05	9
5	F	518.63	546.64	833.94	8
6	S	605.71	633.72	686.76	7
7	G	662.76	690.77	599.68	6
8	K	790.93	818.94	542.63	5
9	P	888.05	916.06	414.46	4
10	A	959.13	987.13	317.34	3
11	A	1030.20	1058.21	246.26	2
12	R	-	-	175.19	1



Display the ion series for charge state: +1 Dta: Gia_Silac_2B_03.1239.1248.2 Precursor mass: 619.48 Mass type: Average

Mod's: (C* +57.022) (R# +6.000)

	AA	A Ions	B Ions	Y Ions	
1	G	30.05	58.06	-	12
2	I	143.21	171.22	1181.34	11
3	V	242.34	270.35	1068.18	10
4	E	371.46	399.46	969.05	9
5	F	518.63	546.64	839.94	8
6	S	605.71	633.72	692.76	7
7	G	662.76	690.77	605.68	6
8	K	790.93	818.94	548.63	5
9	P	888.05	916.06	420.46	4
10	A	959.13	987.13	323.34	3
11	A	1030.20	1058.21	252.26	2
12	R#	-	-	181.19	1



5.2.4.5 Protein comparison view with Mass spectrometric analysis for looking at difference of doublets

5.2.4.5.1 Spectrum comparison

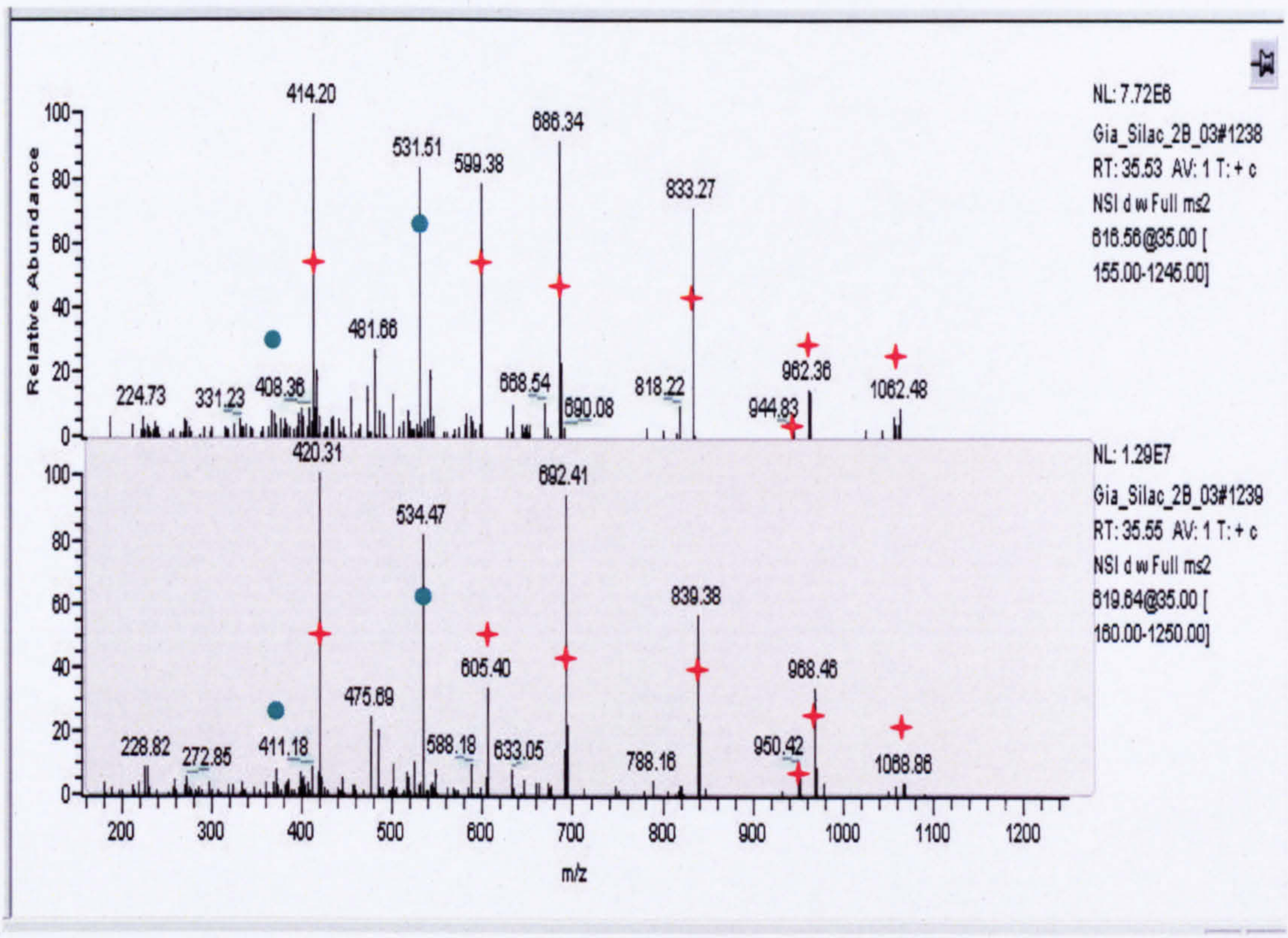
To valuate the matches of heavy and light ions after fragmentation, the data was also analysed using the Xcalibur software package. Figure 5.13 illustrates the coupling of stable isotopes and mass spectrometry for both the identification of proteins and the quantification of their relative levels. Multiple singly and doubly charged peaks in MS/MS spectra from 616m/z and 619 m/z are shown. The corresponding fragmented light and heavy ions differ by 6 m/z units, which represent singly charged ions and by 3m/z units, which represent doubly charged ions.

Figure 5-13 Spectrum comparison

MS/MS data was analysed with Xcalibur software package. The m/z of the ions was determined in the initial MS analysis and was used to select ions for MS/MS analysis. Multiple singly and doubly charged peaks in MS/MS spectra from 616m/z and 619m/z are shown as an example. Corresponding fragmented light and heavy ions are shown which differ by 6 m/z units if were singly charged and 3m/z units if doubly charged. The top panel showed the fragmented light ions and the bottom panel showed the fragmented heavy ions. The paired ions singly charged, are labelled by a red cross. The doubly charged paired ions are marked by a green circle.

5.2.4.5.2 *Ared comparison*

The fragmentation spectra were analyzed and annotated with the Xpress (ThermoFinnigan software programme). This programme facilitates the location of the predicted heavy and light pairs. The data was checked manually to confirm the identification and to verify that no interfering peaks were detected. The results of the paired ions is used to evaluate their abundance. The results of the are shown in Figure 5.14.



5.2.4.5.2 Area comparison

The fragmentation spectra were analyzed and quantified with the Xpress (ThermoFinnigan software programme). This programme calculates the ratio of the predicted heavy and light pairs. The data was checked manually to confirm the identification and to verify that an isotopic pair had been detected. The ratio of the paired ions is used to evaluate their abundance. Examples of Xpress data analysis are shown in Figure 5.14.

Figure 5-14 Area comparison

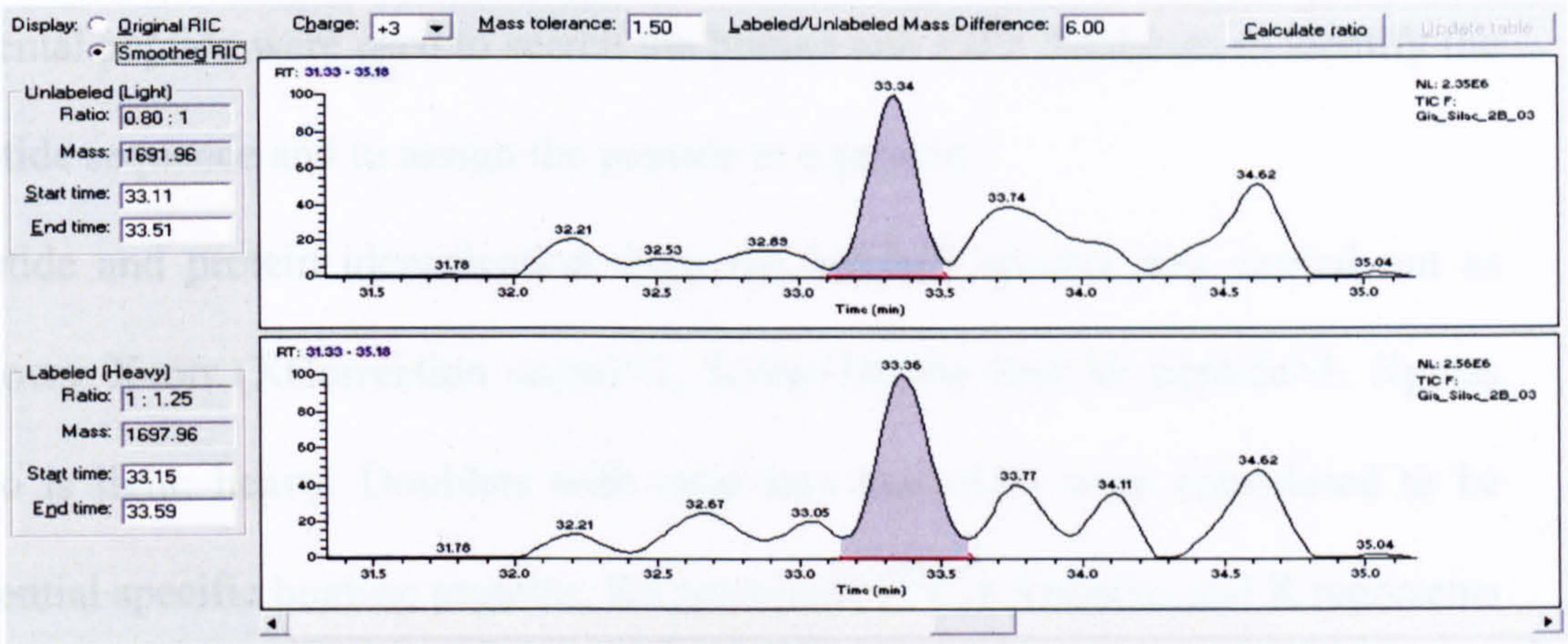
On the left hand side of the each graph are examples of the selection parameters of ions: charge, mass, ratio of light: heavy (upper) or heavy: light (down), start and end time of elution. The shaded peak shows the identified ion. The farther away from 1 the ratio is, the more likely the protein is to interact with LMP2A or LMP2B.

(A) The top panel shows the ratio of the two peptides was close to 1. This represents a non-specifically bound protein.

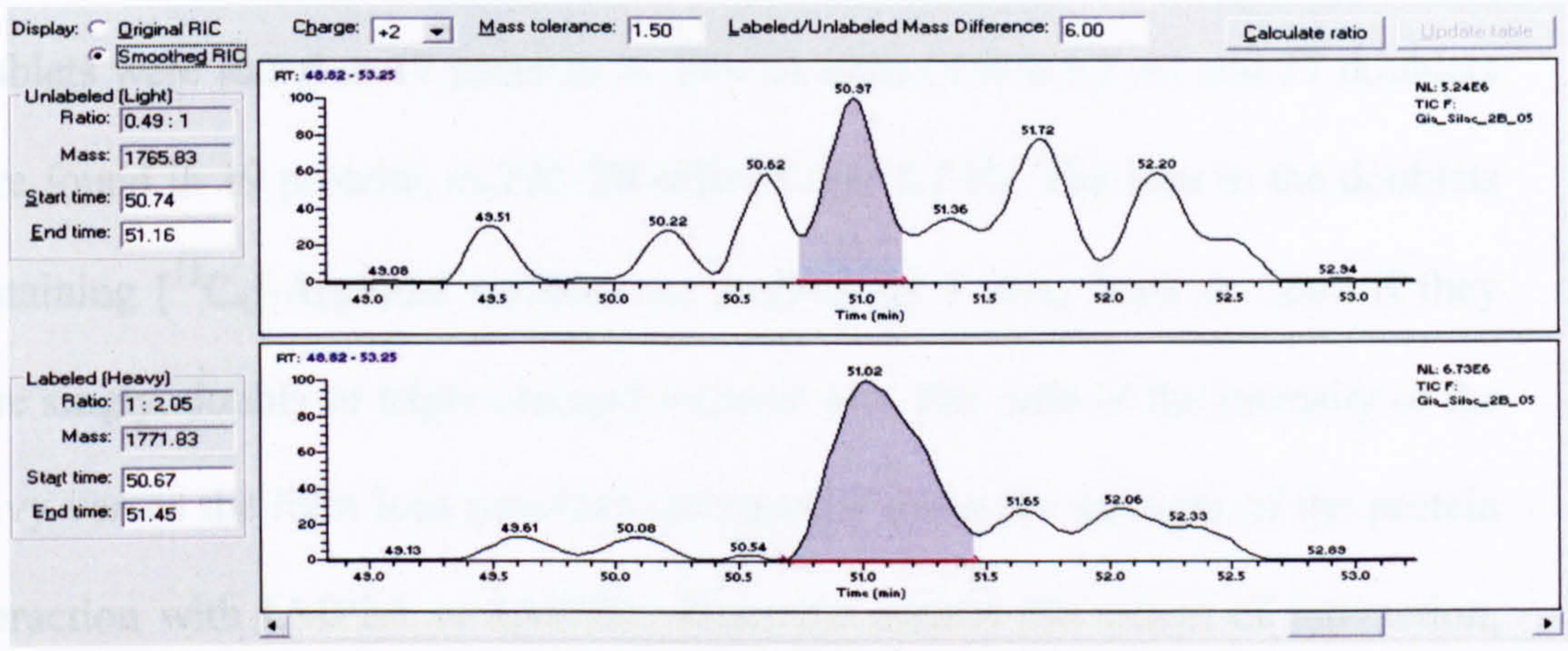
(B) The middle panel shows the heavy peptide to be twice the abundance of the light peptide.

(C) The lower panel shows the heavy peptide to be 10 times more abundant than the light peptide, which are therefore potential interacting proteins.

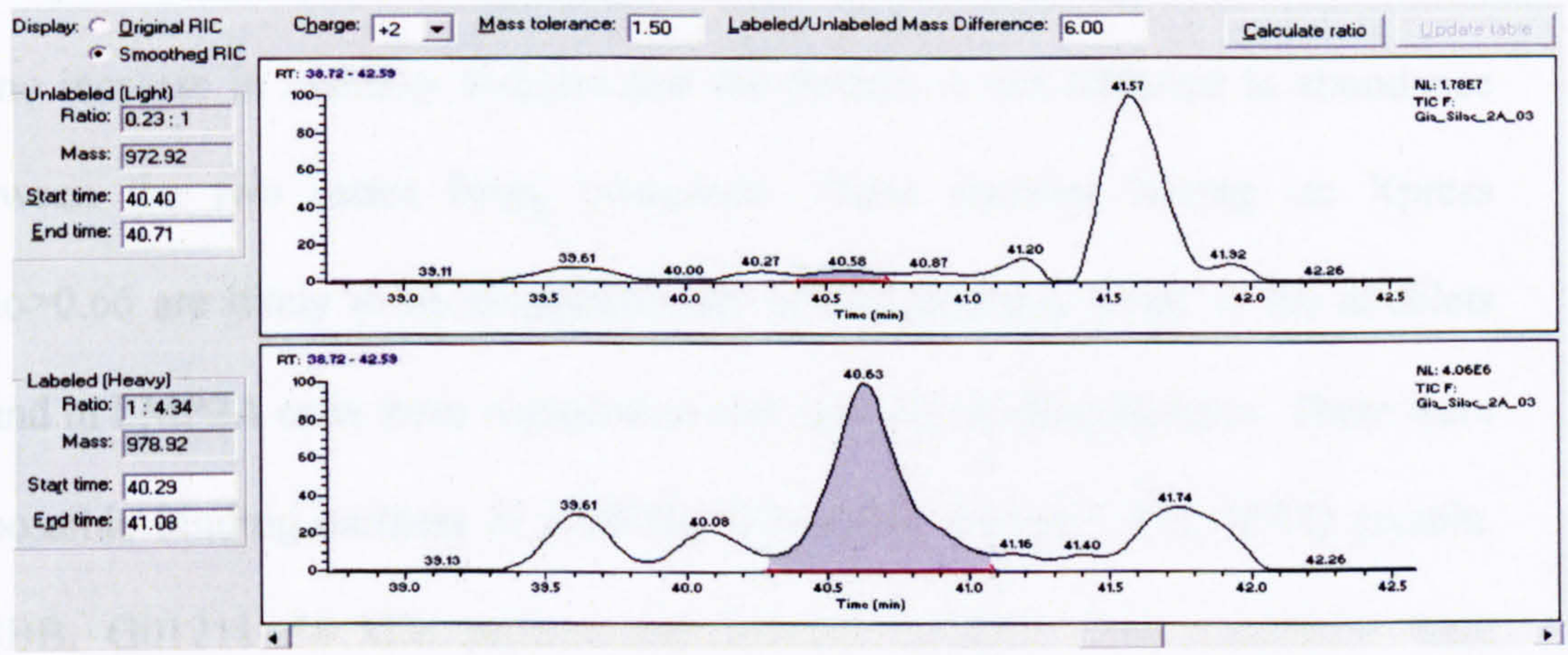
A


Display: Original RIC
Smoothed RIC
Charge: +3
Mass tolerance: 1.50
Labeled/Unlabeled Mass Difference: 6.00
Calculate ratio
Update table
Unlabeled (Light)
Ratio: 0.80 : 1
Mass: 1691.96
Start time: 33.11
End time: 33.51
RT: 31.33 - 35.18
NL: 2.35E6
TIC F:
Gis_Silac_2B_03
33.34
32.21
32.53
32.69
31.78
33.74
34.62
35.04
Time (min)
Labeled (Heavy)
Ratio: 1 : 1.25
Mass: 1697.96
Start time: 33.15
End time: 33.59
RT: 31.33 - 35.18
NL: 2.56E6
TIC F:
Gis_Silac_2B_03
33.36
32.21
32.67
33.05
31.76
33.77
34.11
34.62
35.04
Time (min)

B


Display: Original RIC
Smoothed RIC
Charge: +2
Mass tolerance: 1.50
Labeled/Unlabeled Mass Difference: 6.00
Calculate ratio
Update table
Unlabeled (Light)
Ratio: 0.49 : 1
Mass: 1765.83
Start time: 50.74
End time: 51.16
RT: 48.82 - 53.25
NL: 5.24E6
TIC F:
Gis_Silac_2B_05
50.97
49.51
50.22
50.62
49.08
51.36
51.72
52.20
52.94
Time (min)
Labeled (Heavy)
Ratio: 1 : 2.05
Mass: 1771.83
Start time: 50.67
End time: 51.45
RT: 48.82 - 53.25
NL: 6.73E6
TIC F:
Gis_Silac_2B_05
51.02
49.61
50.08
50.54
49.13
51.65
52.06
52.33
52.69
Time (min)

C


Display: Original RIC
Smoothed RIC
Charge: +2
Mass tolerance: 1.50
Labeled/Unlabeled Mass Difference: 6.00
Calculate ratio
Update table
Unlabeled (Light)
Ratio: 0.23 : 1
Mass: 972.92
Start time: 40.40
End time: 40.71
RT: 38.72 - 42.59
NL: 1.78E7
TIC F:
Gis_Silac_2A_03
41.57
39.61
40.00
40.27
40.58
40.87
41.20
39.11
41.92
42.26
Time (min)
Labeled (Heavy)
Ratio: 1 : 4.34
Mass: 978.92
Start time: 40.29
End time: 41.08
RT: 38.72 - 42.59
NL: 4.06E6
TIC F:
Gis_Silac_2A_03
40.63
39.61
40.08
39.13
41.16
41.40
41.74
42.26
Time (min)

5.2.4.6 Quantification of protein ratios from peptide doublets in 293-2A or 293-2B cells

In the MS, MS/MS analysis, the fragmentation pattern and the mass of the parental peptide were used to search the human and EBV databases to identify the peptide sequence and to assign the peptide to a protein.

Peptide and protein identification from the MS/MS spectra was carried out as follows: Xcorr (XCorrection score)>2, Score>10, the first hit peptide>1. Xpress ratio is light: heavy. Doublets with ratio less than 0.66 were considered to be potential specific binding proteins. R# represents [$^{13}C_6$]-Arginine and R represents [$^{12}C_6$]-Arginine. MH+ represents mass of ion (M: molecule; H+, proton). 20 doublets were found in 17 proteins in 293-2A cells (Table 5.2 A) and 27 doublets were found in 19 proteins in 293-2B cells (Table 5.2 B). The ions in the doublets containing [$^{13}C_6$]-Arginine residues are heavier by 6 m/z, 3m/z or 2m/z if they were singly, doubly or triply charged respectively. The ratio of the intensity of the heavy versus the light ions provides information about the strength of the protein interaction with LMP2A or LMP2B. Thus, the greater the extent of interaction, the higher its abundance in binding to LMP2A or LMP2B. Peptide pairs with little or no increase in intensity indicate that the protein is not different in abundance between the two states being compared. These proteins having an Xpress ratio>0.66 are likely to be nonspecifically bound proteins, so all of the doublets found in LMP2A cells were regarded as non-specific binding partners. There were 5 possible binding partners of LMP2B, Ribosomal protein L27a, SFPQ protein, TUBB, G01211 54 kDa protein and EEF1A1 protein. The remainder were classified as non-specifically bound proteins.

Table 5-2 Quantification of protein ratios from peptide doublets in LMP2A and LMP2B cells

The peptides containing [$^{13}C_6$]-Arginine residues are heavier by 6 Da. The ratio of light versus heavy ions provides information on the degree of these interactions with of LMP2A or LMP2B. The greater the interaction, the higher the ratio. Peptide pairs with a ratio close to 1 indicate that the protein is not different in abundance in the two states being compared and is likely to be interacting non-specifically. Doublets displaying a ratio less than 0.66 were considered to be specific interactions. In the Table, R# represents [$^{13}C_6$]-Arginine and R represents [$^{12}C_6$]-Arginine. MH+ represents mass of ion (M: molecule; H+, proton), and XCorr>2, Score>10, the first hit peptide>1. Xpress ratio is light: heavy.

(A) 20 doublets of 17 proteins that were found in 293-2A cells compared with 293-Zeo cells and all of them are identified as possible non-specific.

(B) 27 doublets of 19 proteins that were found in 293-2B cells compared with 293-Zeo cells. 5 proteins, Ribosomal protein L27a, SFPQ protein, TUBB, G01211 54 kDa protein and EEF1A1 protein showed a ratio<0.66 and can therefore be regarded as specific LMP2b interactions.

A

Name of proteins and Scan(s)	Sequence	MH+	Charge	XC	Score	Accession	Peptides (Hits)	XPRESS
HSPCA protein					**50.24**	**62914009.0**	**5 (5 0 0 0 0)**	
1736 - 1744	-.GVVDSEDLPLNISR.-	1514.66	2	3.21	0.00	1107.4	19/26	
1738	-.GVVDSEDLPLNISR#.-	1520.66	2	2.01	0.08	569.7	14/26	1 : 0.66
heat shock protein 90					**20.19**	**1620018.0**	**2 (2 0 0 0 0)**	
1724	-.HLEINPDHSIIETLR.-	1788.00	3	3.71	0.56	1298.0	26/56	
1726	-.HLEINPDHSIIETLR#.-	1794.00	3	2.85	0.44	853.7	22/56	1 : 0.64
TRAP1					**20.16**	**3273383.0**	**2 (2 0 0 0 0)**	
1736 - 1744	-.GVVDSEDIPLNLSR.-	1514.66	2	3.21	0.13	1107.4	19/26	
1738	-.GVVDSEDIPLNLSR#.-	1520.66	2	2.01	0.00	569.7	14/26	1 : 0.66
heat shock protein 72					**60.22**	**4691418.0**	**6 (6 0 0 0 0)**	
1241 - 1248	-.ATAGDTHLGGEDFDNR.-	1676.68	2	3.26	0.44	845.9	17/30	
1247	-.ATAGDTHLGGEDFDNR#.-	1682.68	3	2.85	0.35	392.6	21/60	1 : 0.47
1859 - 1862	-.IINEPTAAAIAYGLDR#.-	1694.91	2	4.41	0.58	2047.7	24/30	0.74 : 1
1865	-.IINEPTAAAIAYGLDR.-	1688.91	2	4.24	0.51	1634.6	24/30	
p54nrb					**40.15**	**2808511.0**	**4 (4 0 0 0 0)**	
1322 - 1331	-.GIVEFSGKPAAR.-	1232.41	2	2.61	0.41	1012.8	17/22	
1329 - 1337	-.GIVEFSGKPAAR#.-	1238.41	2	2.03	0.31	644.5	14/22	1 : 0.70
tubulin alpha 6 variant					**20.18**	**62897609.0**	**2 (2 0 0 0 0)**	
2144	-.AVFVDLEPTVIDEVR#.-	1708.93	2	3.47	0.40	658.0	18/28	1 : 0.79
2145 - 2151	-.AVFVDLEPTVIDEVR.-	1702.93	2	3.55	0.45	774.3	19/28	
EEF1A1 protein					**40.27**	**39795379.0**	**4 (4 0 0 0 0)**	
1448	-.IGGIGTVPVGR.-	1026.22	2	2.76	0.28	426.3	12/20	
1451	-.IGGIGTVPVGR#.-	1032.22	2	2.29	0.34	443.1	13/20	1 : 0.70
ACTG1 protein					**30.15**	**40225338.0**	**3 (3 0 0 0 0)**	
1238 - 1245	-.QEYDESGPSIVHR#.-	1523.58	3	3.09	0.13	1167.1	23/48	1 : 0.73
1244 - 1250	-.QEYDESGPSIVHR.-	1517.58	3	2.80	0.11	732.7	18/48	

ATDO actin-slime mold					**16.24**	**71629.0**	**2 (0 2 0 0 0)**	
1238 - 1245	-.EEYDESGPSIVHR#.-	1524.57	3	2.68	0.42	815.1	20/48	1 : 0.73
1244 - 1250	-.EEYDESGPSIVHR.-	1518.57	3	2.50	0.25	662.0	17/48	
HSPCA protein					**50.24**	**62914009.0**	**5 (5 0 0 0 0)**	
1736 - 1744	-.GVVDSEDLPLNISR.-	1514.66	2	3.21	0.00	1107.4	19/26	
1738	-.GVVDSEDLPLNISR#.-	1520.66	2	2.01	0.08	569.7	14/26	1 : 0.66
YWHAZ protein					**30.16**	**49119653.0**	**3 (3 0 0 0 0)**	
1178	-.SVTEQGAELSNEER#.-	1555.58	2	3.12	0.27	1295.3	18/26	1 : 0.84
1194	-.SVTEQGAELSNEER.-	1549.58	2	2.91	0.27	666.6	14/26	
myoblast antigen 24.1D5					**90.17**	**23712.0**	**9 (9 0 0 0 0)**	
1100 - 1107	-.FGQGGAGPVGGQGPR.-	1342.45	2	2.96	0.46	1046.7	18/28	
1101 - 1106	-.FGQGGAGPVGGQGPR#.-	1348.45	2	3.16	0.47	1623.3	20/28	1 : 0.83
1268 - 1275	-.FATHAAALSVR.-	1144.31	2	2.76	0.41	1226.0	17/20	
1270 - 1274	-.FATHAAALSVR#.-	1150.31	2	3.40	0.43	1214.8	17/20	0.78 : 1
1366 - 1373	-.FAQHGTFEYEYSQR#.-	1769.85	3	2.84	0.31	679.3	20/52	1 : 0.82
1367 - 1371	-.FAQHGTFEYEYSQR.-	1763.85	3	3.29	0.27	648.5	22/52	
1368 - 1377	-.GIVEFASKPAAR.-	1246.44	2	2.44	0.45	529.2	12/22	
1370 - 1378	-.GIVEFASKPAAR#.-	1252.44	2	2.18	0.43	712.9	13/22	1 : 0.79
SFPQ protein					**30.17**	**33879558.0**	**3 (3 0 0 0 0)**	
2019 - 2024	-.LFVGNLPADITEDEFKR#.-	1971.20	3	3.13	0.19	466.2	23/64	0.82 : 1
2020 - 2025	-.LFVGNLPADITEDEFKR.-	1965.20	3	3.01	0.25	528.1	25/64	
ECH1 protein					**20.19**	**16924265.0**	**2 (2 0 0 0 0)**	
1892 - 1904	-.VIGNQSLVNELAFTAR.-	1732.96	2	3.73	0.44	992.5	17/30	
1895	-.VIGNQSLVNELAFTAR#.-	1738.96	2	2.57	0.38	643.2	14/30	0.91 : 1
mutant beta-actin					**28.20**	**28336.0**	**3 (2 1 0 0 0)**	
1316 - 1323	-.GYSFTTTAER#.-	1139.19	2	2.83	0.44	1048.3	14/18	0.88 : 1
1322 - 1330	-.GYSFTTTAER.-	1133.19	2	2.65	0.46	863.4	14/18	
calnexin					**20.16**	**7709904.0**	**2 (2 0 0 0 0)**	

1875	-.APVPTGEVYFADSFDR.-	1771.91	2	3.25	0.35	882.2	17/30	
1883	-.APVPTGEVYFADSFDR#.-	1777.91	2	2.75	0.35	727.7	16/30	0.79 : 1
Hypothetical protein LOC341883					**20.29**	**38348312.0**	**2 (2 0 0 0 0)**	
1290 - 1298	-.R#IIKVNNR#.-	1025.22	2	2.25	0.02	716.3	11/14	0.21 : 1
1300 - 1305	-.RIIKVNNR.-	1013.22	2	2.31	0.01	793.8	12/14	

B

Name of proteins and Scan(s)	Sequence	MH+	Charge	XC	Score	Accession	Peptides (Hits)	XPRESS
myoblast antigen 24.1D5					**100.18**	**23712.0**	**10 (10 0 0 0 0)**	
1124 - 1130	-.FGQGGAGPVGGQGPR.-	1342.45	2	3.57	0.53	1446.1	20/28	
1131 - 1136	-.FGQGGAGPVGGQGPR#.-	1348.45	2	2.79	0.46	1369.5	20/28	1 : 0.69
1274 - 1282	-.FATHAAALSVR#.-	1150.31	2	3.35	0.27	895.0	15/20	1 : 0.90
1276 - 1281	-.FATHAAALSVR.-	1144.31	2	3.03	0.39	1184.5	17/20	
1376 - 1382	-.FAQHGTFEYEYSQR.-	1763.85	3	3.03	0.28	648.7	22/52	
1380	-.FAQHGTFEYEYSQR#.-	1769.85	2	3.28	0.52	787.4	15/26	1 : 0.77
1706	-.GVVDSEDLPLNISR.-	1514.66	2	3.39	0.00	1105.1	20/26	
1708	-.GVVDSEDLPLNISR#.-	1520.66	2	3.24	0.00	770.1	18/26	0.94 : 1
p54nrb					**80.15**	**2808511.0**	**8 (8 0 0 0 0)**	
1139	-.FAC*HSASLTVR#.-	1255.40	2	2.22	0.38	936.3	14/20	0.95 : 1
1238 - 1246	-.GIVEFSGKPAAR.-	1232.41	2	2.72	0.39	1231.4	17/22	
1239 - 1248	-.GIVEFSGKPAAR#.-	1238.41	2	2.79	0.44	810.2	15/22	1 : 0.64
1240 - 1245	-.GIVEFSGKPAAR.-	1232.41	3	2.69	0.43	438.9	21/44	
1653	-.FAQPGSFEYEYAMR#.-	1702.86	2	2.85	0.39	422.1	15/26	0.93 : 1
1655	-.FAQPGSFEYEYAMR.-	1696.86	2	2.89	0.45	448.3	16/26	
heat shock protein 72					**70.23**	**4691418.0**	**7 (7 0 0 0 0)**	
1144 - 1152	-.ATAGDTHLGGEDFDNR#.-	1682.68	2	4.29	0.52	943.3	19/30	1 : 0.91
1145 - 1151	-.ATAGDTHLGGEDFDNR#.-	1682.68	3	3.04	0.29	682.1	26/60	1 : 0.41
1148 - 1154	-.ATAGDTHLGGEDFDNR.-	1676.68	3	2.51	0.24	484.2	23/60	
1149 - 1155	-.ATAGDTHLGGEDFDNR.-	1676.68	2	3.12	0.43	794.7	17/30	
1772	-.IINEPTAAAIAYGLDR#.-	1694.91	2	3.42	0.34	1445.2	22/30	1 : 0.82
1773 - 1779	-.IINEPTAAAIAYGLDR.-	1688.91	2	4.58	0.50	1248.3	22/30	
p80 protein					**30.16**	**1483131.0**	**3 (3 0 0 0 0)**	
1151	-.VDNDENEHQLSLR#.-	1575.62	3	2.83	0.28	426.6	20/48	1 : 0.47
1160	-.VDNDENEHQLSLR.-	1569.62	2	2.66	0.36	775.7	13/24	

YWHAZ protein					**20.16**	**49119653.0**	**2 (2 0 0 0 0)**	
1178	-.SVTEQGAELSNEER#.-	1555.58	2	3.19	0.36	923.4	17/26	1 : 0.73
1179 - 1186	-.SVTEQGAELSNEER.-	1549.58	2	3.07	0.36	1455.7	20/26	
MGC:128832 protein					**30.20**	**73587131.0**	**3 (3 0 0 0 0)**	
1322	-.TPVEPEVAIHR#.-	1254.41	3	2.87	0.34	1350.2	23/40	1 : 0.86
1324 - 1331	-.TPVEPEVAIHR.-	1248.41	3	2.88	0.16	1667.1	25/40	
ribosomal protein L27a					**20.14**	**4432754.0**	**2 (2 0 0 0 0)**	
1743 - 1748	-.TGAAPIIDVVR#.-	1118.31	2	2.73	0.36	664.0	15/20	0.64 : 1
1749	-.TGAAPIIDVVR.-	1112.31	2	2.63	0.33	863.5	17/20	
SFPQ protein						**33879558.0**	**5 (5 0 0 0 0)**	
1370 - 1378	-.GIVEFASKPAAR.-	1246.44	2	2.72	0.38	863.6	14/22	
1371 - 1379	-.GIVEFASKPAAR#.-	1252.44	3	2.79	0.30	530.6	19/44	0.64 : 1
hnRNP protein A2					**20.14**	**500638.0**	**2 (2 0 0 0 0)**	
950 - 968	-.YHTINGHNAEVR#.-	1417.51	3	2.51	0.33	680.6	17/44	0.91 : 1
957	-.YHTINGHNAEVR.-	1411.51	3	2.84	0.24	1093.3	21/44	
TUBB					**20.24**	**49456871.0**	**2 (2 0 0 0 0)**	
2172	-.SGPFGQIFRPDNFVFGQSGAGNNWAK.-	2800.04	3	3.40	0.38	818.1	28/100	0.58 : 1
2174	-.SGPFGQIFR#PDNFVFGQSGAGNNWAK.-	2806.04	3	4.73	0.53	1531.0	32/100	0.53 : 1
Uracil DNA glycosylase					**60.16**	**35053.0**	**6 (6 0 0 0 0)**	
1850	-.LISWYDNEFGYSNR#.-	1770.88	2	3.24	0.47	1017.8	17/26	0.49 : 1
1853	-.LISWYDNEFGYSNR.-	1764.88	2	2.92	0.36	1047.1	16/26	
G01211 54 kDa protein						**7446345.0**	**3 (3 0 0 0 0)**	
1128 - 1138	-.FAC*HSASLTVR#.-	1255.40	3	2.96	0.50	1054.5	22/40	0.48 : 1
1137	-.FAC*HSASLTVR.-	1249.40	2	2.83	0.44	1061.0	16/20	
unknown					**20.19**	**62702219.0**	**2 (2 0 0 0 0)**	
1395 - 1400	-.LVQDVANNTNEEAGDGTTTATVLAR#.-	2567.70	3	3.84	0.40	513.0	26/96	0.71 : 1

1397	-.LVQDVANNTNEEAGDGTTTATVLAR.-	2561.70	3	3.06	0.33	628.7	26/96	
EEF1A1 protein					**40.15**	**39795379.0**	**4 (4 0 0 0 0)**	
1358 - 1364	-.IGGIGTVPVGR.-	1026.22	2	2.89	0.40	830.3	16/20	
1359 - 1365	-.IGGIGTVPVGR#.-	1032.22	2	3.04	0.50	1078.5	17/20	0.64 : 1
ACTG1 protein					**40.14**	**40225338.0**	**4 (4 0 0 0 0)**	
1118 - 1125	-.QEYDESGPSIVHR.-	1517.58	3	2.84	0.08	803.7	21/48	
1119 - 1126	-.QEYDESGPSIVHR#.-	1523.58	3	2.52	0.05	685.0	18/48	0.99 : 1
1120	-.QEYDESGPSIVHR.-	1517.58	2	2.69	0.23	543.5	13/24	
1121	-.QEYDESGPSIVHR#.-	1523.58	2	2.72	0.18	618.0	13/24	0.99 : 1
mutant beta-actin					**30.19**	**28336.0**	**3 (3 0 0 0 0)**	
1209 - 1214	-.GYSFTTTAER#.-	1139.19	2	2.12	0.08	887.7	13/18	0.79 : 1
1215 - 1221	-.GYSFTTTAER.-	1133.19	2	2.49	0.40	759.2	13/18	
ATDO actin-slime mold					**24.32**	**71629.0**	**3 (0 3 0 0 0)**	
1118 - 1125	-.EEYDESGPSIVHR.-	1518.57	3	2.62	0.23	637.3	19/48	
1121	-.EEYDESGPSIVHR#.-	1524.57	2	2.22	0.39	486.2	11/24	0.99 : 1
TRAP1					**20.17**	**3273383.0**	**2 (2 0 0 0 0)**	
1706	-.GVVDSEDIPLNLSR.-	1514.66	2	3.39	0.15	1105.1	20/26	
1708	-.GVVDSEDIPLNLSR#.-	1520.66	2	3.24	0.38	770.1	18/26	0.94 : 1
hypothetical protein XP_374104					**16.53**	**51464347.0**	**2 (0 2 0 0 0)**	
1706	-.ALALSLSTGPGLGLSR.-	1513.76	2	2.89	0.34	321.2	15/30	
1708	-.ALALSLSTGPGLGLSR#.-	1519.76	2	2.00	0.44	263.4	13/30	0.94 : 1

5.2.4.7 Potential binding partners of LMP2A or LMP2B without finding their doublets

The data by analysis identified one third of the peptides which have [$^{13}C_6$]-Arginine with high Xcorr, first hit peptide and Xpress ratio<0.66, but without detection of the counterpart light peptide. These data were searched manually again using Turbosequest and Xcalibur software package. The new criteria were: 1) Finding light counterpart from unfiltered database. 2) Paired fragments were found manually by Xcalibur software. 3) Xpress ratio <<0.66. 4) Xpress area showed a neat, monopeak with ratio<<0.66. 5) MS/MS showed a clear background with good matches of y-ion and b-ion. From the new search, 10 proteins were found as potential LMP2A binding partners and 15 proteins as potential LMP2B interactions (Table 5.3).

Table 5-3 Potential binding partners of LMP2A and LMP2B without finding their binding partners

The data base was searched manually again using Turbosequest and Xcalibur software package according to the new criteria as described above. 10 novel proteins were found as potential LMP2A binding partners and 15 novel proteins as potential LMP2B interactors, of which particular interest is the possible interaction of DNA methyl transferase 1 (DNMT1) with LMP2A and OB-cadherin with LMP2B.

Name of proteins and Scan(s)	Sequence	MH+	Charge	XC	Score	Accession	Peptides (Hits)	XPRESS
LMP2A								
ORF 61, ribonuleotide reductase large subunit homolog					10.15	2246520.0	1 (1 0 0 0 0)	
1869	-.LPENPDR#CHSLFQGVC*IPTLFFR#.-	2760.15	3	2.97	0.19	459.7	22/88	0.24 : 1
MYST protein 4					10.19	68565921.0	1 (1 0 0 0 0)	
1432 - 1438	-.SQTVAMQGPAR#TLTMQR#.-	1889.18	3	2.55	0.02	357.0	19/64	0.42 : 1
DNA METHYLTRANSFERASE					18.35	5870276.0	2 (1 1 0 0 0)	
1821 - 1826	-.R#C*GVCEVC*QQPECGK.-	1759.97	2	2.45	0.30	173.2	11/28	0.26 : 1
KIAA1454 protein					10.14	7959167.0	1 (1 0 0 0 0)	
1250 - 1256	-.EYEQR#LNELQEER#.-	1748.82	3	2.75	0.17	633.1	19/48	0.16 : 1
immunoglobulin heavy chain variable					10.13	37777891.0	1 (1 0 0 0 0)	
1241 - 1248	-.TAYMELSSLR#NDDTAVYYC*AR#.-	2512.72	3	2.56	0.10	339.5	19/80	0.06 : 1
KIAA0713 protein					10.18	3882147.0	1 (1 0 0 0 0)	
1413	-.FYPSFR#QNALYVR#.-	1673.89	2	2.15	0.07	491.4	13/24	0.13 : 1
PREDICTED: hypothetical protein XP_373750					10.11	51474101.0	1 (1 0 0 0 0)	
1162 - 1166	-.GGGGGAASGER#AVLGGR#.-	1441.53	2	2.22	0.12	216.8	11/32	0.20 : 1
truncated calcitonin/calcitonin-related polypeptide, alpha					10.13	67515411.0	1 (1 0 0 0 0)	
1454 - 1460	-.FSPFLALSILVLLQAGSLHAAPFR#.-	2576.07	3	2.64	0.09	277.3	19/92	0.03 : 1
Kruppel-like factor 15					10.11	7662494.0	1 (1 0 0 0 0)	
1658	-.FSR#SDELSR#.-	1109.16	2	2.29	0.21	233.5	9/16	0.09 : 1
KIAA1909 protein					10.26	71891715.0	1 (1 0 0 0 0)	

1557	-.DEFIVCCGR#KK.-	1304.56	2	2.02	0.21	438.5	10/20	0.37 : 1
LMP2B								
T42658 hypothetical protein DKFZp434K0115.1					10.33	11360071.0	1 (1 0 0 0 0)	
1142 - 1148	-.AR#GNLELR#PGGAHPGTC*SPSR#.-	2209.39	2	2.72	0.14	251.3	12/40	0.01 : 1
OB-cadherin-1					10.25	1377894.0	1 (1 0 0 0 0)	
1072 - 1078	-.TALPNMDR#EAKEEYHVVIQAK.-	2449.77	3	2.60	0.24	348.5	20/80	0.61 : 1
CG7793-PA					10.14	22946434.0	1 (1 0 0 0 0)	
2072 - 2079	-.ELQMKVEHWEDKDVGQNC*NEFIR#.-	2912.17	3	2.90	0.15	262.6	17/88	0.08 : 1
FUN14 domain containing 2					10.08	57209640.0	1 (1 0 0 0 0)	
1491 - 1497	-.SNQIPTEVR#SK.-	1265.40	1	1.66	0.01	322.5	10/20	0.05 : 1
RGPG542					10.12	37182157.0	1 (1 0 0 0 0)	
1247 - 1251	-.VEIVPR#DLR#.-	1109.29	2	2.39	0.16	448.3	11/16	0.08 : 1
unnamed protein product					20.14	897763.0	2 (2 0 0 0 0)	
1244	-.ISVYYNEATGGK.-	1302.42	2	2.51	0.31	950.8	16/22	
1887	-.YLTVAAVFR#.-	1046.24	2	2.89	0.38	958.9	14/16	0.33 : 1
EF1a-like protein					20.14	12006049.0	2 (2 0 0 0 0)	
1542 - 1564	-.YYVTIIDAPGHR#.-	1411.58	2	2.81	0.41	1164.2	16/22	0.53 : 1
1703 - 1712	-.EHALLAYTLGVK.-	1315.54	2	2.45	0.42	1199.3	18/22	
KIAA1454 protein					10.13	7959167.0	1 (1 0 0 0 0)	
1146 - 1160	-.EYEQR#LNELQEER#.-	1748.82	3	2.59	0.10	617.7	20/48	0.34 : 1
Ribosomal protein SA					10.12	47125390.0	1 (1 0 0 0 0)	
1202	-.FAAATGATPIAGR#.-	1210.36	2	2.37	0.25	1297.7	17/24	0.41 : 1
PGAM1					20.16	49456447.0	2 (2 0 0 0 0)	
1236 - 1240	-.HYGGLTGLNK.-	1060.19	2	2.76	0.39	901.9	16/18	
1341	-.HGESAWNLENR#.-	1319.36	2	3.19	0.30	1053.1	15/20	0.47 : 1
unnamed protein product					16.14	16553072.0	2 (1 0 1 0 0)	

1811 - 1814	-.NATMIFGILASNNDVKKLLR#.-	2225.64	3	2.78	0.08	512.7	21/76	0.08 : 1
KIAA0690 protein					10.16	3327194.0	1 (1 0 0 0 0)	
1853 - 1857	-.GEDEEMADPMEDVIIR#NKK.-	2226.47	2	3.16	0.08	378.7	12/36	0.03 : 1
janus kinase 1					10.11	68299593.0	1 (1 0 0 0 0)	
1790	-.LVNTLKEGKR#.-	1164.38	2	2.18	0.20	328.4	11/18	0.28 : 1
Kruppel-like factor 15					10.11	7662494.0	1 (1 0 0 0 0)	
1700	-.FSR#SDELSR#.-	1109.16	2	2.12	0.20	237.7	9/16	0.43 : 1
HIST1H4F					10.19	49457374.0	1 (1 0 0 0 0)	
1244 - 1252	-.DNIQGITKPAIR#.-	1332.53	2	2.53	0.42	529.0	13/22	0.58 : 1

5.3 Discussion

LMP2 is expressed in most EBV-associated tumours, including Hodgkin's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma (Brooks L., *et al*, 1992; Busson P, *et al.*, 1992; Niedobitek *et al.*, 1997; Takada K *et al.*, 2000). Some proteins have been reported to interact with LMP2A in different cell backgrounds, but no systematic identification of LMP2 interacting proteins has been attempted. In this study, His-tagged LMP2A and LMP2B genes were expressed in HEK 293 cells and quantitative SILAC analysis with LC-MS/MS analysis was performed to identify potential LMP2 interacting proteins.

SILAC has recently emerged as a valuable proteomic technique that has the capability to identify specific protein-protein interactions (Ibarrola *et al.*, 2003, Ibarrola *et al.*, 2004, Blagoev *et al.*, 2003). Mann and colleagues used this approach in HeLa cells to examine changes associated with epidermal growth factor (EGF) signalling (Blagoev *et al.*, 2003). Coupled with affinity purification to assess specific protein-protein interactions, their studies resulted in the identification and relative quantification of 228 proteins; 28 of which showed a significant enrichment upon stimulation by EGF with the rest being non-specific interactions.

Here SILAC identified 10 potential interactors for LMP2A and 20 potential interactors for LMP2B. However, all of these require validation by other techniques such as immunoprecipitation and immunofluorescence. Of the potential interactors identified several are worthy of further discussion. DNA Methyl Transferase (DNMT) was identified as a potential LMP2A interactor. DNMTs include DNMT1, DNMT3a and DNMT3b. Overexpression of DNMT1 has been detected in several human cancers (Sun *et al.*, 1997; Saito *et al.*, 2001;

Kanai *et al.*, 2001). It was reported that DNMT1 increased cancer cell proliferative activity and may be associated with EBV infection and other etiological factors during gastric carcinogenesis (Etoh *et al.*, 2004). EBV itself controls virus gene expression through the methylation of virus gene (Paulson and Speck, 1999). Furthermore, it has been shown that LMP1 downregulates expression of E-cadherin gene by activating of DNA methyltransferases (Tsai *et al.*, 2002). It will be interesting to explore the potential cooperation between DNA methylation and signalling pathways mediated by LMP2.

OB-cadherin was identified here as a possible LMP2B-interacting protein; this protein is widely distributed and functions as a cell-to-cell signalling and adhesion molecule, most notably in bone, where it is found on the bone cells that constantly remodel and maintain the structural integrity of bone (Okazaki *et al.*, 1994; Lecanda et *al.*, 2000; Kii *et al,* 2004). OB-cadherin is important in certain mechanisms of tumour spread. Many reports suggest that OB-cadherin plays a significant role in cancer (Pishvaian *et al.*, 1999; Tomita *et al.*, 2000; Nagi *et al.*, 2005). Early-stage carcinomas do not express OB-cadherin, but rather express E-cadherin, like the epithelial cells from which they are derived. However, cadherin switching may occur where OB-cadherin is up-regulated as the tumour dedifferentiates, facilitating metastatic dissemination by promoting the motility of the tumour cells (Cavallaro *et al.*, 2002; Hazan *et al.*,2004; Suyama *et al.*, 2002). It was reported that LMP2B may directly influence cell adhesion and motility in epithelial cells (Allen *et al.*, 2005). More importantly, the array results (Chapter 4) show that LMP2B altered a great proportion of genes involved in adhesion and motility in KMH2 cells. Therefore it will be important to investigate whether LMP2B interaction with OB-cadherin is involved in this process.

SILAC combined with LC-MS/MS to identify protein interactors is a long process. Any steps could have an influence to the results. Several aspects affect to identify specific interacting proteins. Keratin contamination happens very frequently, which interferes with LC-MS/MS analysis to identify some important but less abundant peptides by occupying the most hit proteins. Purification of LMP2 proteins is a possible step to lose LMP2 interacting proteins. Handling the proteins gently to avoid disrupt LMP2's interacting proteins is the key. Getting rid of the non-specific proteins, which compete binding sites of the Ni-NTA beads increases the possibilities to identify the specific ones.

It is concluded that the combination of LC-MS/MS and SILAC is an excellent, reliable method in quantitative proteomics to identify interacting proteins of LMP2. This study has successfully shown that the technology, SILAC can be applied to a model of identifying interacting proteins. Extending from this, SILAC can be applied to various researches for which SILAC could prove useful for quantitative proteomic analysis.

CHAPTER SIX: CONCLUSIONS AND FUTURE WORK

6 Conclusions and future work

The transfection of HL cell lines is always regarded as problematic and possibly more so if the transgenes are toxic transmembrane proteins such as LMP2. An alternative way to deliver expression of LMP2 to EBV-negative HL cell lines is by the use of retroviral vectors carrying these viral genes. Therefore, the effect of LMP2A and LMP2B in HL cell background is able to be investigated.

The expression of LMP2 in the two infected HL cell lines showed a similar pattern, which was low at two weeks post infection, gradually increasing and remaining stable at 8 weeks post-infection. Both proliferation and viability assays showed no significant difference with the expression of LMP2A or LMP2B in KMH2 and L428 cells as compared with their control neomycin-expressing counterparts at two weeks post-infection. At two weeks post-infection, the toxicity of LMP2A might predominate over its effect on proliferation, particular in the low serum conditions used or the expression of LMP2 may have been insufficient to induce the proliferation of HL cells. This result does not exclude the possibility that LMP2 provides a survival signal to HL cells, particularly as gene expression microarrays showed that the genes commonly altered by LMP2A and LMP2B are mainly involved in signalling transduction, proliferation and anti-apoptosis regulation. Therefore, it would be worthwhile to perform the proliferation and viability assays with the LMP2 expressing cells at 8 weeks post infection to further explore the growth and survival ability of LMP2.

Analysis of the microarray data from LMP2A and LMP2B expressing KMH2 cells identified many changes in cellular gene expression, some of which were examined in more detail. Semi-quantitative PCR confirmed the array result that IFI27, an interferon–α regulated gene implicated in cancer, was down-regulated

by LMP2A, but not LMP2B. The down regulation of IFI27 was also observed in EBV infected KMH2 cells compared with its parental counterparts and L591 cells compared with their EBV-negative counterparts. However, how LMP2A or EBV down-regulate IFI27 in HL cell lines and what the functional consequences of this down-regulation are need further investigation. LMP2A might maintain EBV latency and provide its survival signals for HRS cells through down-regulating IFI27. Experiments examining the signalling pathways responsible for LMP2A-induced down-regulation of IFI27 using LMP2A mutants and/or chemical inhibitors (e.g. blockade of PI3-kinase) should be performed. These could be compared to the effects of directly reducing IFI27 expression using RNA interference. The expression of IFI27 in fresh HL biopsies should also be performed and a comparison between EBV-positive and EBV-negative HL cases undertaken.

The expression level of LMP2 appeared to influence cellular gene expression. AICDA was down-regulated in KMH2 cells expressing lower levels of LMP2A, KMH2 cells infected with EBV, and EBV positive L591 cells. In contrast, late passage KMH2-LMP2A cells expressing higher levels of LMP2A up-regulated AICDA. Thus it appears that 'physiological' levels of LMP2A are important in determining the gene expression consequence of this protein. Therefore, it will be necessary to confirm the RNA expression change described here at the protein level and to determine how this relates to the level of LMP2A expression in HRS cells.

The loss of B cell lineage markers by HRS cells has been widely reported. In this study, many genes involved in BCR signalling that are down-regulated in HL,

were found to be up-regulated by LMP2A. This suggests that LMP2A utilises some components of the BCR pathway to maintain EBV latency, possibly to transform GC cells into HRS cells, but the precise details of how LMP2A uses BCR signalling docking proteins to play its role needs to be investigated. The use of LMP2A mutants with specific changes in key signalling motifs should help to further dissect the pathways involved in regulating specific aspects of cellular gene expression.

To identify cellular proteins that interact with LMP2, His-tagged versions of LMP2A and LMP2B were stably expressed in HEK 293 cells. The expression of LMP2 in these cells was confirmed. The His-tag of LMP2B (sited at the N-terminus) was partially hidden in the native condition because of the confirmation of this transmembrane protein. In future studies the effect of a C-terminal His-tag should be examined. The combination of LC-MS/MS and SILAC is a reliable method in quantitative proteomics to identify interacting proteins of LMP2. Using this approach, 10 LMP2A potential binders and 20 LMP2B potential binders were identified. All these potential interactions require validation but of particular interest is the possible interaction of DNA methyl transferase 1 (DNMT1) with LMP2A and OB-cadherin with LMP2B. It has been reported that DNMT1 can increase cancer cell proliferative activity and may be associated with EBV infection, so it will be interesting to explore the potential cooperation between DNA methylation and signalling pathways mediated by LMP2. Of particular importance is the detailed validation of the interaction between LMP2A and DNMT1 including an examination of specificity (using LMP2A mutants) and a thorough analysis of subcellular localisation of the two proteins.

OB-cadherin is widely distributed and functions as a cell-to-cell signalling and adhesion molecule, functional effects that may be induced by LMP2B. Gene expression arrays showed that LMP2B altered a large number of genes associated with adhesion and motility in HL cells, one of which (cadherin13) is related to OB-cadherin. Detailed validation of this interaction, particularly as it appears to be occurring with a membrane spanning molecule, is necessary including co-localisation studies using confocal microscopy. Once confirmed, functional studies examining the effects of LMP2B on cadherin-mediated effects such as cell adhesion and motiliy could be performed.

Overall , the studies described in this thesis demonstrate that LMP2A and LMP2B have profound effects on the behaviour of HL cells and thus are likely to contribute to the role of EBV in the pathogenesis of HL. In future studies it will be important to move away from the use of HL cell lines and develop an in vitro model that will allow an assessment of LMP2 function in a more appropriate cellular environment. We have recently validated an approach in which EBV genes are transfected into isolated GC B cells and any effects are subsequently examined using microarray analysis. It will be interesting to use this method to determine the effect of LMP2A and LMP2B on the primary GC B cell phenotype and to relate this to the data presented in this thesis.

CHAPTER SEVEN: REFERENCES

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CHAPTER EIGHT: APPENDIX

8 Appendix

8.1 Sequences of primers

8.1.1 Primers for PCR

LMP2A (full length):

Forward primer: 5'-ATG ACT CAT CTC AAC ACA TAT ACG AAG AAG-3'
Reverse primer: 5'-ATA AAA ACT GGA CCG TAT GTT CTA TTT CCA-3'

LMP2A (N-terminus):

Forward primer: 5'-ATG ACT CAT CTC AAC ACA TA-3'
Reverse primer: 5'-CAT GTT AGG CAA ATT GCA AA-3'

LMP2B (full length):

Forward Primer: 5'- ATG TAT GAA TCC AGT ATG CCT GCC T-3'
Reverse Primer: 5'-TTA TAC AGT GTT GCG ATA TGT CCA-3'

8.1.2 Primers for making constructs

LMP2A

KpnI-Forward 5'-CGGGGTACCTATGGGGTCCCTAGAAATGG-3'
Xbal-Reverse 5'-CGGTCTAGATTATACAGTGTTGCGATATG-3'

LMP2B

KpnI-Forward 5'-CGGGGTACCTATGAATCCAGTATGCCTGCCT-3'
Xbal-Reverse 5'-CGGTCTAGATTATACAGTGTTGCGATATG-3'

LMP2A-N

KpnI-Forward 5'-CGGGGTACCTATGGGGTCCCTAGAAATGG-3'
Xbal-Reverse 5'-CGGTCTAGATTAACTTCCTCTGCCCGCTTCTTCGT-3'

8.2 Media, solutions and reagent

8.2.1 Solutions for small scale and large scale extraction of plasmid DNA

Solution I (100ml)

50mM Glucose 0.9g
25mM TrisHCl (PH 8.0) 0.3g
10mM EDTA (PH 8.0) 0.37g
Autoclaved for 15 min at 10p/sg.
Store at 4°C.

Solution II:

0.2N NaOH (freshly diluted from 10N NaOH)
1% SDS

Solution III(100ml):

60ml 5M potassium acetate
11.5ml Glacial acetic acid
28.5ml H_2O

8.2.2 L-Broth Agar solution and L-Broth Agar plate

L-Broth Agar solution:

10g L-Broth powder
7.5gNutrient Agar
500 ml distilled H_2O
Autoclaved. Add Amipicillin after it cools down (1mg/ml).

L-Broth plate solution

10g L-Broth powder

500 ml distilled H_2O
Autoclaved. Add Amipicillin after it cools down (1mg/ml).

8.2.3 Solution for western blotting

PBS-Tween

100 Dulbecco A tablets (Oxoid Ltd)
10 ml Tween 20 (Sigma, P7949).
10 litre distilled H_2O

TBS-Tween
24.2g Tris
80 NaCl
10ml Tween-20
10 litre distilled H_2O
pH 7.6

Gel Sample Buffer (GSB)
50mM Tris solution (pH6.8)
4% SDS (w/v)
10% glycerol
5%2-mercaptocethanol
0.01% bromophenol.

Running buffer
30g Tris
144g glycine
10g SDS
10 litre distilled H_2O
pH 8.3-8.8.

Transfer buffer
30g Tris
144g of glycine
2 litres of methanol
8 litres of distilled H_2O

RIPA buffer
50mM Tris pH 8.0
150mM NaCl
1% NP-40
0.5% deoxycholate
0.1% SDS in SDW
5µg/ml leupeptin (Sigma, L8511)
Aprotinin (Sigma, p5318)
0.5mM sodium orthovanadate (Sigma, s6508)
0.5mM sodium fluoride (Sigma, S7920)
1mM PMSF (Sigma, P7626) added immediately proior to use.

8M Urea Lysis Buffer
Dissolved 480.48 urea (8M) in 1 litre dH2O

Stripping Buffer
100mM Tris-HCl pH 6.8
2% (w/v) SDS
50mM 2-mercaptoethanol

8.2.4 Solution for immunofluorescence staining

4% Paraformaldehyde (PFA)

20g PFA dissolved in 500 ml distilled H_2O by stirring overnight at 42°C.
Once dissolved, 5 Dulbecco A were added.

20%HINGS

Heat inactivated normal goat serum diluted to 20% in PBS.

DABCO

90ml of glycerol
2.5g of DABCO power
10 ml PBS
pH 8.6
Covered in silver foil

8.2.5 Buffers for purification of His-tagged proteins

Lysis buffer:

50mM NaH_2PO
300mM NaCl
10mM imidazole
1% Triton-X-100
pH 8.0

Wash buffer:

50mM NaH_2PO
300mM NaCl
20-40mM imidazole
pH 8.0

Elution buffer:

50mM NaH_2PO
300mM NaCl
250mM imidazole
pH 8.0

8.2.6 Buffers for gene expression microarray

12X MES Stock:

64.61g MES hydrate
193.3g MES Sodium salt
800 ml RNAse free dH_2O
Mix and adjust volume to 1000ml. Check the pH is between 6.5 and 6.7.
Filter through a 0.2μm filter.

2X Hybridisation Buffer (50ml):

8.3 ml of 12X MES Stock Buffer
17.7 ml of 5M NaCl
4.0 ml of 0.5M EDTA
0.1 ml of 10% Tween 20
19.9 ml RNase free dH_2O
Store at 4^0C

2X Stain Buffer (250ml):

41.7 ml 12X MES Stock Buffer
92.5 ml 5M NaCl
2.5 ml 10% Tween 20
112.8 ml RNase free dH_2O

Filter through a 0.2μm filter and store at 4^0C in the dark.

Non-Stringent Wash Buffer (A) (1000ml):

300 ml 20 X SSPE
1 ml 10% Tween 20
698 ml dH_2O
Filter through a 0.2μm filter.

Stringent Wash Buffer (B) (1000ml):

83.3 ml 12X MES Stock Buffer
5.2 ml 5M NaCl
1 ml 10% Tween 20
910.5 ml dH_2O
Filter through a 0.2μm filter and store at 4^0C in the dark

8.2.7 Buffer for staining

Coomassie Brilliant Blue

0.08% Coomassie Brilliant Blue G250
1.6% Orthophosphoric Acid
8% Ammonium Sulphate
20% Methanol

8.3 List of genes altered by LMP2A or LMP2B in KMH2 cells

Table 8-1: Classification of cellular genes up- and down-regulated by LMP2A

Gene symbol	Gene name	Fold change	References
Anti-apoptosis			
BDNF	brain-derived neurotrophic factor	1.7	PMID: 15781614 PMID: 16181428 PMID: 10962554 PMID: 15604270
SORBS1	sorbin and SH3 domain containing 1	2.8	PMID: 15504914 PMID: 15569975
DAPK1	death-associated protein kinase 1	2.5	PMID: 16142356 PMID: 10378697
MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	2.1	PMID: 16198555 PMID: 12816952
CA8	carbonic anhydrase VIII	1.8	PMID: 15069192
DCT	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2)	3	PMID: 12695295 PMID: 12505051
GBA3	glucosidase, beta, acid 3 (cytosolic)	2.7	PMID: 12077779 PMID: 12901861
KLF5	Kruppel-like factor 5 (intestinal)	1.8	PMID: 16046710 PMID: 12637494
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	1.8	PMID: 15590693
RBMS3	RNA binding motif, single stranded interacting protein	1.9	PMID: 10675610 PMID: 15086514
RTN1	reticulon 1	2	PMID: 15350194
UBD	ubiquitin D	2	PMID: 11445583 PMID: 11557113
API5	Apoptosis inhibitor 5	1.7	PMID: 10780674
C3orf1	Chromosome 3 open reading frame 1	1.6	PMID: 15126327 PMID: 9018111
CHN2	Chimerin (chimaerin) 2	-2.7	PMID: 9103432
CST11	cystatin 11	-3.1	PMID: 10099331
GAGE2,4, 5,6,7,7B,8	G antigen 2,4,5,6,7,7B,8	-2.4	PMID: 12432251 PMID: 12432252 PMID: 15987717
GSPT1	G1 to S phase transition 1	1.7	PMID: 15917414
IMPA2	inositol(myo)-1(or 4)-monophosphatase 2	1.7	PMID: 14767552
KIAA0367	KIAA0367	1.8	PMID: 16288218
NMT2	N-myristoyltransferase 2	1.7	PMID: 16123142 PMID: 15700311
RPRM	reprimo, TP53 dependant G2 arrest mediator candidate	-4.9	PMID: 11313928
SEMA6A	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	-2.2	PMID: 10993894
UNC13B	unc-13 homolog B (C. elegans)	-1.6	PMID: 10233166 PMID: 12655055
UNC5C	unc-5 homolog C (C. elegans)	1.6	PMID: 11387206 PMID: 11479215
COL1A1	collagen, type I, alpha 1	1.6	PMID: 15727634 PMID: 16107717
MAGED1	melanoma antigen family D, 1	1.7	PMID: 15911347
Cytoskeleton			
ARHGAP6	Rho GTPase activating protein 6	1.5	PMID: 10699171 PMID: 12697302
FRMD4A	FERM domain containing 4A	-2	PMID: 16086323 PMID: 11598191
KIAA0992	palladin	-1.6	PMID: 16164966 PMID: 15716039
PLEKHC1	pleckstrin homology domain containing, family C (with FERM domain) member 1	1.6	PMID: 15927810 PMID: 15686479
SCIN	scinderin	2.4	PMID: 12438125
Differentiation			
CPNE4	copine IV	-3.4	PMID: 10416158 PMID: 10935488
FABP4	fatty acid binding protein 4, adipocyte	-1.9	PMID: 15862280
GPM6A	glycoprotein M6A	1.7	PMID: 12359212 PMID: 15530461
HEBP1	heme binding protein 1	1.8	PMID: 15530461

KIAA1202	KIAA1202 protein	-1.8	PMID: 16249884
LASS6	LAG1 longevity assurance homolog 6 (S. cerevisiae)	-1.8	PMID: 16211262
SERPINE2	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.6	PMID: 10449603
TM7SF1	Transmembrane 7 superfamily member 1 (upregulated in kidney)	1.7	PMID: 9521871
TRA2A	Transformer-2 alpha	1.6	PMID: 15210204
SPANXB1,B2	SPANX family, member B1/B2	3	PMID: 14734458
SOX9	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	-2	PMID: 12173687 PMID: 10412367
Immunity			
SASH1	SAM and SH3 domain containing 1	1.9	PMID: 16137664
IGKC /// IGKV1-5	immunoglobulin kappa constant /// immunoglobulin kappa variable 1-5	2.4	PMID: 1961033
FCRLM1	Fc receptor-like and mucin-like 1	1.7	PMID: 16176992
GBP1	guanylate binding protein 1, interferon-inducible, 67kDa /// guanylate binding protein 1, interferon-inducible, 67kDa	1.6	PMID: 10087221 PMID: 12396730 PMID: 15717119
GBP2	guanylate binding protein 2, interferon-inducible	1.5	PMID: 11726669
IFI27	interferon, alpha-inducible protein 27	-2.9	PMID: 15661146 PMID: 16210605
IL23R	interleukin 23 receptor	1.6	PMID: 15912962
SEPP1	selenoprotein P, plasma, 1	-2.8	PMID: 8562289 PMID: 16380510
AICDA	activation-induced cytidine deaminase	8.8	PMID: 16180254
FCRLM1	Fc receptor-like and mucin-like 1	1.7	
IGKC /// IGKV1-5	immunoglobulin kappa constant /// immunoglobulin kappa variable 1-5	2.4	PMID: 12357370
Metabolism			
CYP4X1	cytochrome P450, family 4, subfamily X, polypeptide 1	-2	PMID: 15797250 PMID: 15752708 PMID: 16191478
DPYS	dihydropyrimidinase	-1.8	PMID: 16176880 PMID: 16338408
FARSLB	phenylalanine-tRNA synthetase-like, beta subunit	1.7	PMID: 15000680 PMID: 15804423
FSIP1	fibrous sheath interacting protein 1	1.6	PMID: 15860413 PMID: 15796179
MGST1	microsomal glutathione S-transferase 1	1.6	PMID: 15366924
NAPSB	napsin B aspartic peptidase pseudogene	-1.7	PMID: 14674886 PMID: 16007166
TPO	thyroid peroxidase	-1.9	PMID: 12536361 PMID: 16200454
ACPL2	acid phosphatase-like 2	1.8	PMID: 14585503 PMID: 12899827
ARG99	ARG99 protein	-3.6	PMID: 14555658 PMID: 16243809
CYP4Z1	cytochrome P450, family 4, subfamily Z, polypeptide 1	-3.3	PMID: 15797250 PMID: 15265682
DCT	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2)	1.9	PMID: 16033338 PMID: 12697670
FSTL5	follistatin-like 5	-4.7	PMID: 12039070
GDPD1	Glycerophosphodiester phosphodiesterase domain containing 1	1.6	PMID: 14612981 PMID: 9441746
GPR39	G protein-coupled receptor 39	2.5	PMID: 16284170
RAB27B	RAB27B, member RAS oncogene family	5.6	PMID: 15713878 PMID: 16314394
SNAP25	synaptosomal-associated protein, 25kDa	1.7	PMID: 15895198 PMID: 15754005
SULT1C1	sulfotransferase family, cytosolic, 1C, member 1	1.9	PMID: 11740338 PMID: 11906176
SULT1C1	sulfotransferase family, cytosolic, 1C, member 1	2.1	PMID: 15754005
Motility			
RGS13	regulator of G-protein signalling 13	1.8	PMID: 15047827 PMID: 15313557
ADAMTS2	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 2	2.2	PMID: 14505115
FRMD4A	FERM domain containing 4A	-2.2	PMID: 10718198
GBP5	Guanylate binding protein 5	2.3	PMID: 15311059

IGSF3	immunoglobulin superfamily, member 3	1.7	PMID: 11673522
ITIH5	inter-alpha (globulin) inhibitor H5	-1.8	PMID: 14744536
LOXL2	lysyl oxidase-like 2	1.7	PMID: 12154058 PMID: 16096638
MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	-1.9	PMID: 12970751 PMID: 15552795 PMID: 15659660
TJP4	Tight junction protein 4 (peripheral)	1.8	PMID: 12407192
TRIB2	tribbles homolog 2 (Drosophila)	1.7	PMID: 15311059
WASF3	WAS protein family, member 3	1.6	PMID: 15907837 PMID: 12185600 PMID: 16061841
PRL	prolactin	-2.3	PMID: 15645137 PMID: 12782572 PMID: 12138195 PMID: 8034749
DSC1	desmocollin 1	-2.8	PMID: 15998767 PMID: 9214392
TIAM1	T-cell lymphoma invasion and metastasis 1	-2.1	PMID: 12134164 PMID: 15340013
JAM3	junctional adhesion molecule 3	-1.9	PMID: 12953056 PMID: 11823489
COL21A1	collagen, type XXI, alpha 1	-2.8	PMID: 11863369 PMID: 11827793
Proliferation			
APP	amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease)	-2.8	PMID: 15637681 PMID: 11557776
FGF13	fibroblast growth factor 13	-2.1	PMID: 15814652 PMID: 16223856
GAGE2, 4,5,6,7,7B	G antigen 2,4,5,6,7,7B	-2.2	PMID: 14656993
IL6R	interleukin 6 receptor	2.2	PMID: 15982327 PMID: 12200683
PRG4	proteoglycan 4	-1.8	PMID: 15719068
TRIM9	tripartite motif-containing 9	2	PMID: 16007190
Signal transduction			
PIAS1	Protein inhibitor of activated STAT, 1	1.5	PMID: 11788578 PMID: 15311277 PMID: 15489189 PMID: 15297606
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	1.7	PMID: 16024605 PMID: 16042555
EPS8	epidermal growth factor receptor pathway substrate 8	2.7	PMID: 14699156 PMID: 9087167
TNFRSF17	tumor necrosis factor receptor superfamily, member 17	2.1	PMID: 16116167 PMID: 15294946
ARHGAP18	Rho GTPase activating protein 18	1.6	PMID: 15492870
AXIN2	axin 2 (conductin, axil)	1.5	PMID: 15735151 PMID: 15572025
BLNK	B-cell linker	1.9	PMID: 11489945 PMID: 15029213
BMP10	bone morphogenetic protein 10	-1.7	PMID: 16049014
BRDG1	BCR downstream signaling 1	2.2	PMID: 10518561 PMID: 12540842
CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)	2.5	PMID: 15763430 PMID: 15817459 PMID: 9584199
EZH2	Enhancer of zeste homolog 2 (Drosophila)	1.9	PMID: 15882624
FAT3	FAT tumor suppressor homolog 3 (Drosophila)	1.7	PMID: 16059920
GPM6A	glycoprotein M6A	1.6	PMID: 12359212
GPR125	G protein-coupled receptor 125	1.8	PMID: 15203201
KCTD3	potassium channel tetramerisation domain containing 3	2	PMID: 11152963
KIAA1671	KIAA1671 protein	3.2	PMID: 15289310
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1 /// leucine-rich repeats and immunoglobulin-like domains 1	2.5	PMID: 15871317
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	1.7	PMID: 15833743
MEGF10	MEGF10 protein	1.7	PMID: 11347906
NYREN18	NEDD8 ultimate buster-1	1.7	PMID: 16171779 PMID: 15347646

PREX1	phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	-1.7	PMID: 15750623 PMID:10395944
PTPRD	Protein tyrosine phosphatase, receptor type, D	-2	PMID: 9531590
RGS1	regulator of G-protein signalling 1	1.7	PMID: 16160139
SORCS3	sortilin-related VPS10 domain containing receptor 3	1.6	PMID: 15009648 PMID: 15313890
SPRY2	sprouty homolog 2 (Drosophila)	2.3	PMID: 15342396
SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	-1.6	PMID: 15328020
Ufm1	Ubiquitin-fold modifier 1	1.7	PMID: 15071506
WBP5	WW domain binding protein 5	1.9	PMID: 15548568
RIN2	Ras and Rab interactor 2	1.9	PMID: 15574778 PMID: 15965503
TSPAN7	tetraspanin 7	2.3	PMID: 10985391
SNF1LK2	SNF1-like kinase 2	1.7	PMID: 15067358
PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1) /// phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	1.7	PMID: 16170333 PMID: 16169459
DMD	dystrophin (muscular dystrophy, Duchenne and Becker types)	1.9	PMID: 15757977
RAPH1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	1.6	PMID: 11965544 PMID: 15609301 PMID: 16026476
CALM2	Calmodulin 2 (phosphorylase kinase, delta)	1.5	PMID: 11454062 PMID: 15878968
ESRRG	estrogen-related receptor gamma	-1.5	PMID: 15149736 PMID: 15504914
ETV5	ets variant gene 5 (ets-related molecule)	1.8	PMID: 15082862 PMID: 15081108
HEPH	hephaestin	1.5	PMID: 15825077
HRASLS3	HRAS-like suppressor 3	1.5	PMID: 11973642
LCAT	lecithin-cholesterol acyltransferase	1.5	PMID: 14973367 PMID: 11773597 PMID: 15037619
PHLDA1	pleckstrin homology-like domain, family A, member 1	1.6	PMID: 15574883 PMID: 16156666
ELK1	ELK1, member of ETS oncogene family	-1.8	PMID: 16239230

Transcription factors

MYCL1	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	1.9	PMID: 12651874 PMID: 16177095
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	-1.7	PMID: 15121845 PMID: 16297338
ELAVL2	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	2.2	PMID: 15990917 PMID: 15060621
GLULD1	glutamate-ammonia ligase (glutamine synthase) domain containing 1	1.6	PMID: 16169192 PMID: 15208637
IRF2BP2	interferon regulatory factor 2 binding protein 2	1.6	PMID: 12799427
ITM2A	integral membrane protein 2A	2.3	PMID: 11863357 PMID: 16303770
KLF13	Kruppel-like factor 13	1.6	PMID: 12244575
LDB2	LIM domain binding 2	1.7	PMID: 15380350
NEDD9	neural precursor cell expressed, developmentally down-regulated 9	1.8	PMID: 9498705 PMID: 12080349
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	-1.6	PMID: 15897878 PMID: 12145335
NFIB	Nuclear factor I/B	1.7	PMID: 15632069 PMID: 11882901
RNF12	Ring finger protein 12	1.7	PMID: 10431247 PMID: 11604498
TCERG1	transcription elongation regulator 1	-1.6	PMID: 15456888 PMID: 15145061
THRAP2	Thyroid hormone receptor associated protein 2	1.6	PMID: 14638541
VDP	Vesicle docking protein p115	1.6	PMID: 10858540
ZNF91	zinc finger protein 91 (HPF7, HTF10)	1.6	PMID: 11470777 PMID: 14993235
SOX6	SRY (sex determining region Y)-box 6	1.6	PMID: 12677004 PMID: 12549914
SFRS6	splicing factor, arginine/serine-rich 6	-1.8	PMID: 15604250
C9orf4	chromosome 9 open reading frame 4	3.2	PMID: 10603000

			PMID: 16354715
SOX2OT	SOX2 overlapping transcript (non-coding RNA)	2.5	PMID: 15910596
Transport protein			
MGC33887	hypothetical protein MGC33887	10.3	
MOSC2	MOCO sulphurase C-terminal domain containing 2	-1.6	PMID: 12832761
SLC16A6 /// LOC440459	solute carrier family 16 (monocarboxylic acid transporters), member 6	1.7	PMID: 15331564 PMID: 12351693
SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	2.8	PMID: 15197647 PMID: 16128593
SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	1.7	PMID: 16103098
SNX7	sorting nexin 7	2	PMID: 16130112
DNA/RNA metabolism			
FAM9B	family with sequence similarity 9, member B	-1.6	PMID: 12213195 PMID: 16102754
---	CDNA FLJ36544 fis, clone TRACH2006378	1.8	PMID: 15077182
PSME4	Proteasome (prosome, macropain) activator subunit 4	1.6	PMID: 15653075
TCEAL7	transcription elongation factor A (SII)-like 7	3.5	
Unknown			
C15orf27	chromosome 15 open reading frame 27	1.8	
C1orf150	chromosome 1 open reading frame 150	-1.9	
C21orf42	chromosome 21 open reading frame 42	-2.8	
C2orf3	Chromosome 2 open reading frame 3	1.6	
C6orf118	chromosome 6 open reading frame 118	-2.7	
C6orf54	chromosome 6 open reading frame 54	1.7	
C8orf31	chromosome 8 open reading frame 31	1.6	
C9orf70	chromosome 9 open reading frame 70	1.7	
CST11	cystatin 11	-2.6	
CXorf33	chromosome X open reading frame 33	1.8	
FAM45A	family with sequence similarity 45, member A	1.6	
FAM46C	family with sequence similarity 46, member C	2.1	
FLJ34077	Weakly similar to zinc finger protein 195	1.6	
KIAA0776	KIAA0776	1.6	
KIAA1815	KIAA1815	1.6	
LOC200230	similar to KIAA0386	1.6	
NAV2	neuron navigator 2	1.7	
ZBTB8	zinc finger and BTB domain containing 8	1.6	
ZNF227	zinc finger protein 227	1.8	
ZNF567	zinc finger protein 567	1.7	
ZNF638	Zinc finger protein 638	1.7	
ZNF652	zinc finger protein 652	1.6	
DKFZP434J0113	hypothetical protein DKFZp434J0113	-1.7	
FBXO22	F-box protein 22	1.5	
FLJ21986	hypothetical protein FLJ21986	-2.3	
FLJ21986	hypothetical protein FLJ21986	-2.2	
FLJ22457	hypothetical protein FLJ22457	-1.6	
FLJ31810	Hypothetical protein FLJ31810	2.5	
KLHL13	kelch-like 13 (Drosophila)	3.7	
LOC151261	hypothetical LOC151261	1.9	
LOC284757	hypothetical protein LOC284757	1.9	
LOC284757	hypothetical protein LOC284757	1.9	
LOC553137	hypothetical LOC553137	-2	
MGC22265	Hypothetical protein MGC22265 /// Similar to bA110H4.2 (similar to membrane protein)	1.7	
RP1-32F7.2	hypothetical protein FLJ37659	1.7	
SH3GLP3	LOC440460	1.6	
SMYD2	SET and MYND domain containing 2	1.7	
TMCC3	Transmembrane and coiled-coil domains 3	-1.5	
TRIB2	tribbles homolog 2 (Drosophila)	2.2	
UHMK1	U2AF homology motif (UHM) kinase 1	-1.7	
WDR72	WD repeat domain 72	2.6	

Table 8.2: Classification of cellular genes up- and down-regulated by LMP2B

Gene Symbol	Gene Title	Fold change	References
Apoptosis			
DCT	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2)	2.8	PMID: 12695295
GBA3	glucosidase, beta, acid 3 (cytosolic)	2.8	PMID: 12505051 PMID: 12077779
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	2.1	PMID: 11195467 PMID: 16201851
RTN1	reticulon 1	1.9	PMID: 15086514 PMID: 15350194
SESN2	sestrin 2	1.8	PMID: 12203114
UBD	ubiquitin D	2.5	PMID: 11445583
DAPK1	death-associated protein kinase 1	2.0	PMID: 15569975 PMID: 16142356
TCEAL7	transcription elongation factor A (SII)-like 7	4.4	
Cytoskeleton			
PLEKHC1	pleckstrin homology domain containing, family C (with FERM domain) member 1	1.8	PMID: 15716039 PMID: 15927810
KLHL13	kelch-like 13 (Drosophila)	5.7	PMID: 15870933
Differentiation			
SERPINE2	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.9	PMID: 10449603
TSGA2	testis specific A2 homolog (mouse)	1.6	PMID: 15215198
Immunity			
LRIG2	Leucine-rich repeats and immunoglobulin-like domains 2	1.7	PMID: 15145052
SPANXB1 /// SPANXB2	SPANX family, member B1 /// SPANX family, member B2	2.4	PMID: 12393489
AICDA	activation-induced cytidine deaminase /// activation-induced cytidine deaminase	2.2	PMID: 16380510 PMID: 16180254
Metabolism			
NOX1	NADPH oxidase 1	1.7	PMID: 10485709 PMID: 15780757
CYP4Z1	cytochrome P450, family 4, subfamily Z, polypeptide 1	-3.3	PMID: 16243809 PMID: 15797250
FSTL5	follistatin-like 5	-2.8	PMID: 12697670 PMID: 12039070
SULT1C1	sulfotransferase family, cytosolic, 1C, member 1	2.5	PMID: 11906176 PMID: 15754005
CYP4Z1	cytochrome P450, family 4, subfamily Z, polypeptide 1	1.5	PMID: 15059886 PMID: 16243809
FLJ31547	carboxylesterase-like urinary excreted protein	1.8	PMID: 16029166 PMID: 12401131
Motility			
CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	1.3	PMID: 16322224 PMID: 10446804
GBP5	Guanylate binding protein 5	1.8	PMID: 15311059
OTOR	otoraplin	1.6	PMID: 12592021 PMID: 11813878
SPANXA1/B1/A2/XC/B2	sperm protein associated with the nucleus, X-linked, family member A1/B1/A2/C/B2	2.5	PMID: 14734458
OPCML	opioid binding protein/cell adhesion molecule-like	1.6	PMID: 16115914 PMID: 14596858
CDH13	cadherin 13, H-cadherin (heart)	2.5	PMID: 16013438 PMID: 14729458
INHBE	inhibin, beta E	2.1	PMID: 15601830 PMID: 14641267
LAMA3	laminin, alpha 3	1.8	PMID: 7533763 PMID: 15953081
Proliferation			
RUNX1T1	Runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	1.6	PMID: 15728845 PMID: 15331666
GAS6	Growth arrest-specific 6	1.7	PMID: 15605394 PMID: 14667825
Signal transduction			

SORBS1	sorbin and SH3 domain containing 1	2.3	PMID: 15604270 PMID: 15504914
CA2	carbonic anhydrase II	2.0	PMID: 10860835 PMID: 11382925
CNKSR3	CNKSR family member 3	2.4	PMID: 15908431 PMID: 15383320
CTH	cystathionase (cystathionine gamma-lyase)	1.9	PMID: 11711533
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	1.8	PMID: 11683410
RAB39B	RAB39B, member RAS oncogene family	2.0	PMID: 12438742
SPRY2	sprouty homolog 2 (Drosophila)	2.8	PMID: 11053437 PMID: 15313890
WBP5	WW domain binding protein 5	2.0	PMID: 15548568
TSPAN7	tetraspanin 7	2.0	PMID: 15735753 PMID: 15342396
Transcripition factors			
LDB2	LIM domain binding 2	1.8	PMID: 15380350
DPCR1	diffuse panbronchiolitis critical region 1	1.7	PMID: 1347043
Transport protein			
SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	2.0	PMID: 16103098
DNA metabolism			
CXorf26	chromosome X open reading frame 26	1.2	
GPR50	G protein-coupled receptor 50	1.7	PMID: 9933574
MGC16121	zinc finger protein 19 (KOX 12) /// zinc finger protein 23 (KOX 16)	1.6	
ZNF227	zinc finger protein 227	1.7	
Unknown			
MGC33887	hypothetical protein MGC33887	10.1	
FLJ31810	Hypothetical protein FLJ31810	2.7	
MGC16121	hypothetical protein MGC16121	1.6	
MGC39325	hypothetical protein MGC39325	1.9	

* The genes highlighted by red are altered by both LMP2A and LMP2B